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Prevention of Postoperative Adhesions in the Dog by Intravenous Injections of Plasminogen Activators.* (21921)

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Recent studies have indicated that experimental venous thrombi in the rabbit(1), and arterial thrombi in the dog(2) can be successfully liquefied *in vivo* by the intravenous administration of plasminogen activators.[‡] Activation of animal plasminogen was carried out in the above studies by large amounts of streptokinase (SK). In addition, in the dog, plasminogen activation was carried out by injections of a mixture of small amounts of SK and a partially purified human plasminogen preparation(2). This latter method was based on the observation that the activation of animal plasminogen by SK was markedly facilitated by the presence of small amounts of human plasminogen preparations(5).§

The purpose of this report is to present evidence that, in dogs with an experimental

traumatic peritonitis, intravenous injection of large amounts of streptokinase alone, or of a mixture of small amounts of SK and a human plasminogen preparation was capable of preventing or modifying the production of postoperative abdominal adhesions.

Materials and methods. Sixty-three mongrel dogs weighing from 10 to 15 kg were used in these experiments. A traumatic hemorrhagic fibrinous peritonitis was produced by scarifying a 2 x 2 inch area of peritoneal wall with a vegetable grater according to the technique of Wright and associates(6). Immediately following surgery, each animal was injected intramuscularly with 1,000,000 units of a long acting penicillin (Bicillin®). The animals were divided into 3 groups: 23 controls, 32 animals injected intravenously with SK|| alone; and 8 animals injected intravenously with a mixture containing 25,000 units of SK and 60 mg of a partially purified human plasminogen preparation.¶ Of the animals receiv-

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‡ Plasminogen refers to the naturally occurring precursor of plasmin, the proteolytic enzyme of mammalian plasma(3). Other terminology, in current use, includes profibrinolysin for the precursor and fibrinolysin for the active enzyme(4).

§ While 200 units of SK per ml of dog plasma was required to activate significant amounts of plasminogen, the addition of minute amounts of human plasminogen preparations to dog plasma reduced the SK requirement to approximately 20 units per ml of dog plasma(2).

|| A commercial preparation (Varidase) Lot No. 7-1089-413, containing streptokinase and streptodornase, and obtained through the courtesy of Dr. J. M. Rueggsegger of Lederle Laboratories. This preparation also contains streptococcal hyaluronidase as well as other contaminating substances.

¶ A lyophilized human plasminogen preparation prepared from human plasma Fraction III (Cohn) by the Christensen and Smith technic(7). The preparation contained 4% nitrogen and had a proteolytic assay, following streptokinase activation, of 4.2 casein units per mg nitrogen(8).

TABLE I. Summary of Results of I.V. Plasminogen Activators on Prevention of Postoperative Adhesions in Dogs.

Animals	No. of dogs	Results*†		
		No. with dense adhesions	No. with moderate adhesions	No. with no adhesions
Controls	23	19	4	0
SK alone—total group	32	10	5	17
400,000 u. od x 6	2	1	1	0
300,000 u. bid x 2	7	4	0	3
300,000 u. bid x 3	12	5	2	5
400,000 u. bid x 3	11	0	2	9
SK + human plasminogen prep.‡	8	0	6	2

* Classified according to thickness, firmness of adherence, and extent of adhesions as follows: Dense—thick, firmly adherent and covering entire area of abrasion. Moderate—thin, more loosely adherent, and scattered. None—occasional filmy to no adhesions.

† Dividing the entire data into 2 classes (*i.e.*, adhesions vs. no adhesions) and employing the chi square test (R. x 2 table; using Yates' correction) statistical analysis showed a highly significant difference between all treatment groups together as compared to the control animals (P less than .01).

Because it was believed that a trend was present relating SK dosage to suppression of adhesion formation, chi square testing of the controls against the combined 3 lower daily dose levels and against the highest daily dose level was carried out. Results indicated that the first 3 treatment groups combined, differed from the control in a manner to which little significance could be ascribed ($P = 0.05$). The last treatment group however showed a highly significant difference from the control (P less than .001).

The authors are indebted to Dr. Stanley Lang for the statistical treatment of the data.

‡ Injection mixture contained 25,000 u. SK and 60 mg of partially purified human plasminogen preparation.

ing SK alone, 2 were given a single injection of 400,000 units of SK daily for 6 days; 7 received 300,000 units twice daily for 2 days; 12 were injected with 300,000 units twice daily for 3 days; and 11 were given 400,000 units twice daily for 3 days. The animals receiving the activation mixture of SK plus the human plasminogen preparation were given single daily injections for 3 days. Treatment with the plasminogen activators was begun on the first postoperative day. Venous specimens of whole blood were obtained just prior to and 10 minutes following each injection of the plasminogen activator. The specimens were incubated at 37°C in a water bath and observed for clotting and subsequent lysis of the clot. The animals were sacrificed 12 days postoperatively and the extent and nature of the adhesions were noted. Specimens of the traumatized site, heart, lungs, kidneys, liver and spleen were taken for histological study.

Results. A summary of the results is shown in Table I. All of the 23 control animals developed adhesions, dense in 19, and moderate in 4. In the group of 32 animals receiving

SK alone, 17, or approximately half, developed no adhesions, 5 had moderate, and 10 had dense adhesions. The prevention of adhesions was related to the daily dose of SK rather than the duration of treatment, with the best results occurring with the highest dose level of SK. Nine of 11 animals, receiving 400,000 units twice daily for 3 days, did not develop any adhesions. In the group of 8 animals receiving the mixture of 60 mg of the partially purified human plasminogen preparation and 25,000 units of SK, 2 did not develop adhesions, and the other 6 developed moderate adhesions. None of the animals of this latter group exhibited dense adhesions.

In order to define more clearly the effect of I.V. streptokinase on the peritoneal exudate, 3 control animals, and 3 animals receiving 400,000 units SK bid for 3 days, were sacrificed on the fifth postoperative day and the abraded sites examined histologically. Each of the control animals showed an extensive fibrinous exudate with evidence of rapidly developing organization. The exudate in each of the SK-treated animals was minimal with little evidence of organization. The striking

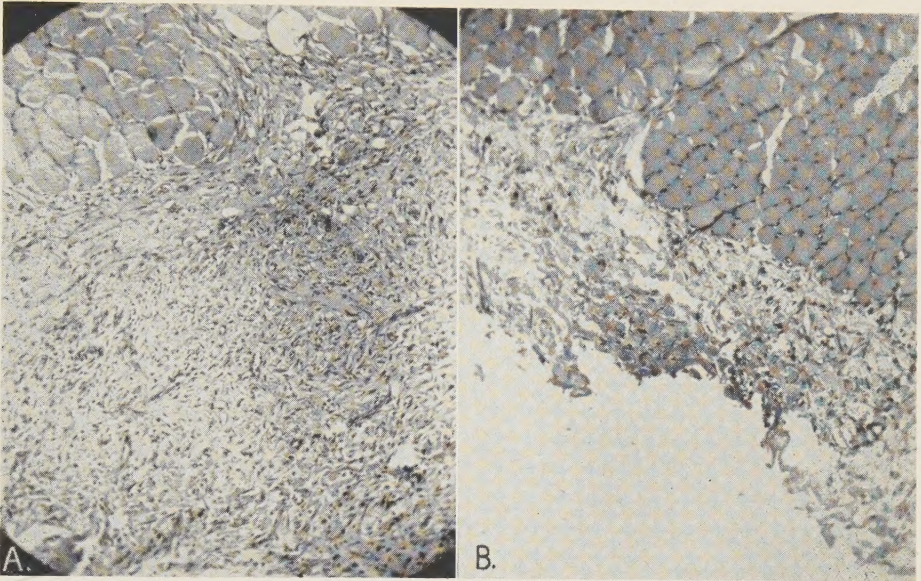


FIG. 1. Effect of I.V. SK on experimental traumatic peritonitis in the dog (see text). Specimens taken from abraded area on 5th postoperative day. A. Control animal. Note thick organizing granulation tissue adjacent to striated muscle fibers in cross section with penetration between muscle bundles (magnification $67\times$). B. SK treated animal. Thin layer of granulation tissue with little evidence of organization (same magnification).

histological difference in a control as compared to an SK-treated animal is shown in Fig. 1.

Each of the blood specimens removed prior to the injection of the activators clotted and did not significantly lyse over an observation period of 48 hours. Following the injection of SK alone, the blood clotted normally and subsequently lysed completely in from one to 4 hours.** Following injection of the mixture of SK and the human plasminogen preparation, the blood either did not clot, or the clot was poorly formed and rapidly lysed over the next 30 minutes. In several instances, determinations were made of plasma fibrinogen, plasminogen, and circulating proteolytic activity, with results similar to those previously reported with these 2 methods of plasminogen activation(2).

A finding of interest observed in the animals receiving SK alone related to the laparotomy wound. Preliminary studies revealed a high incidence of wound dehiscence in animals re-

ceiving large doses of SK alone (300,000 units bid). The dehiscence was attributed to an accelerated resorption of the catgut sutures. With the use of silk sutures, as utilized in the studies reported, no wound dehiscence was noted. However, in the animals treated with the largest daily dose of SK alone (400,000 units bid), fresh hemorrhage into the incisional wound was observed during the 3 days of SK therapy. Following the cessation of SK administration, the hemorrhage rapidly resorbed. At the time of final exploration, the wounds were well healed and did not differ from the control wounds.

No other significant toxic manifestations were noted in any of the animals. All the animals survived the total observation period. Studies of the sections of heart, lung, kidney and liver were not remarkable.†† In the majority of the animals, sections of the spleen revealed passive congestion but the presence of congestion was independent of treatment.

Discussion. The prevention of postopera-

** Lysis times of 1-2 hours were observed after the injection of 400,000 units of SK and 3-4 hours after the injection of 300,000 units of SK.

†† We are indebted to Dr. Philip Wasserman, Department of Clinical Laboratories, Jewish Hospital of Cincinnati, for these studies.

tive abdominal adhesions in many of the animals receiving the intravenous injection of plasminogen activators probably indicates a lysis of the traumatic inflammatory exudate. It is highly unlikely that the effects of the plasminogen activators can be entirely attributed to a lack of formation of a fibrinous exudate since therapy was not instituted for a full day following trauma. The presence of increased capillary permeability in the traumatic area may account for the appearance of plasmin or of SK at the site of exudation. Although the streptokinase preparation contained streptodornase and streptococcal hyaluronidase, both of which may contribute to the lysis and resorption of the exudate, we have stressed the fibrinolytic system since fibrin is the major insoluble component of the experimental exudate in this study.

The differences observed with the 2 types of activators used in this study, and the special fibrinolytic activity seen after *in vivo* injections of SK alone, have been previously noted (2). Recent studies indicate that the mixture of SK plus the human plasminogen preparation supplies the activator for the animal plasminogen(5,9). The special fibrinolytic effects seen with large doses of SK alone are related to the adsorption of SK on fibrin, and the ability of SK to suppress antiplasmin ac-

tivity(10). The resultant of these latter phenomena is to provide an "inhibitor free" fibrin for the action of activated plasmin(10).

Conclusion. In dogs with an experimental traumatic peritonitis, the intravenous injection of streptokinase alone in large doses, or of a mixture of smaller amounts of SK plus a partially purified human plasminogen preparation, prevented or modified the development of postoperative adhesions.

1. Johnson, A. J., and Tillett, W. S., *J. Exp. Med.*, 1952, v95, 449.
2. Sherry, S., Titchener, A., Gottesman, L., Wasserman, P., and Troll, W., *J. Clin. Invest.*, 1954, v33, 1303.
3. Christensen, L. R., and MacLeod, C. M., *J. Gen. Physiol.*, 1945, v28, 559.
4. Loomis, E. C., George, C., Jr., and Ryder, A., *Arch. Biochem.*, 1947, v12, 1.
5. Sherry, S., *J. Clin. Invest.*, 1954, v33, 1054.
6. Wright, L. T., Smith, D. H., Rothman, M., Quash, E. T., and Metzger, W. I., *Proc. Soc. Exp. Biol. and Med.*, 1950, v75, 602.
7. Christensen, L. R., and Smith, D. H., Jr., *ibid.*, 1950, v74, 840.
8. Remmert, LeM., and Cohen, P. P., *J. Biol. Chem.*, 1949, v181, 431.
9. Troll, W., and Sherry, S., *ibid.*, 1955, v213, 881.
10. Sherry, S., in preparation.

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Electrophoretic Distribution of Hexosamine in Plasma Proteins of the Rat Following Thyroidectomy.* (21922)

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Plasma hexosamine levels increase in the rat following either thyroidectomy or hypophysectomy(1). The cause appears to be a deficiency of thyroid hormone in both instances. Hexosamine is present in all plasma protein fractions but is richest in the globu-

lins(2). Since globulins have been reported to be elevated in rats following thyroidectomy (3), electrophoretic studies were carried out to establish which protein fractions were responsible for the plasma hexosamine changes observed in the rat following thyroidectomy.

Methods. Male Sprague-Dawley rats, weighing 55-65 g, were individually caged, fed a high calcium diet(4), and permitted to drink water *ad libitum*. Thyroidectomy was per-

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TABLE I. Electrophoretic Distribution of Plasma Proteins and Hexosamine in Plasma Proteins of Normal and Thyroidectomized Rats.*

Group	Albumin	Globulins				Total
		α -1	α -2	β	γ	
Protein (% of total)						
Control	31.8 \pm 0.2	18.1 \pm .7	14.0 \pm .3	17.5 \pm .7	18.6 \pm 1.3	—
Thyroidectomy	29.7 \pm 1.0	16.3 \pm .8	12.7 \pm .6	18.8 \pm 1.1	22.6 \pm 1.0	—
Significance (P)	<.10	<.15	<.10	<.40	<.03	
Hexosamine (mg/100 ml of plasma)						
Control	5.7 \pm 0.8	48.2 \pm 3.1	21.4 \pm .7	19.6 \pm 1.3	9.7 \pm 1.4	104 \pm 4.8
Thyroidectomy	8.8 \pm 1.1	50.6 \pm 2.4	21.3 \pm .7	28.6 \pm 2.1	18.8 \pm 1.5	128 \pm 4.6
Significance (P)	<.05	>.50	>.50	<.01	<.01	<.01
Hexosamine (% of protein)						
Control	.30	4.42	2.55	1.87	0.87	1.73

* Mean \pm stand. error. Each group contained 6 rats.

formed under ether anesthesia. All rats were weighed on the day of operation and at 12 and 35 days following the day of operation. The thyroidectomized rats used for study were selected on the basis of plateaued growth curves, a measure of complete removal of the thyroid gland. Blood samples were obtained 35 days after the day of operation from the external jugular vein under light ether anesthesia, using oxalate as an anticoagulant. Hematocrit and hexosamine determinations were carried out as previously described, with plasma hexosamine levels being corrected for hematocrit changes(4,5). Electrophoretic separation of the plasma proteins was carried out on filter paper in an apparatus similar to that used by Durrum(6). Whatman No. 1 paper sheets or strips with a resistance area of approximately 500 cm² were used. Electrophoretic separations were carried out at room temperature, at constant current (10mA) in pH 8.6 veronal buffer (ionic strength 0.06) for 11-16 hours. The voltage varied with conditions of evaporation in the system. For protein studies 0.01-0.02 ml of plasma were applied on a 25 mm line. For hexosamine studies 0.1 ml was applied to a 150 mm line. At the completion of a run the paper was dried in an oven at 110°C for 20 minutes, stained for 7 minutes in an alcoholic solution of 0.1% bromphenol blue saturated with mercuric chloride(6), rinsed in tap water and dried again in an oven at 110°C. For protein studies the strip was read at 600 m μ in a Beckman spectrophotometer (model DU) by means of a specially constructed strip holder. A protein distribution curve was obtained by

charting optical density (ordinate) against distance migrated (abscissa). The protein distribution was measured by cutting out the charted electrophoretic curve, weighing it and relating the weight of each component to the total weight.[†] The electrophoretic distribution of hexosamines was determined by cutting the paper according to the known protein bands. Each band of paper was hydrolyzed in 3 ml of 1N hydrochloric acid for 15 hours and after isolation on Dowex-50, hexosamine was determined by a modification of the method of Elson and Morgan(5).

Results. The distribution of plasma proteins following thyroidectomy is shown in the upper part of Table I. There was a significant increase in the relative amount of gamma globulin. The beta fraction was unaltered but the albumin and alpha fractions were relatively reduced. An example of the changes seen following thyroidectomy is shown in Fig. 1.

The concentration of total plasma hexosamine increased following thyroidectomy (middle part of Table I). This change could

[†] The dye-binding capacities of albumin and globulins differ from one another(7) so that the protein values are not those one would get with moving boundary electrophoresis, where albumin values run higher and globulins lower. It was shown in these experiments that the measurements used followed the Beer-Lambert law. This was established by scanning stained strips prepared by electrophoretically separating serum proteins of known concentration. It was shown that the areas (as determined by O. D. readings) under each band were proportional to the original concentrations of protein.

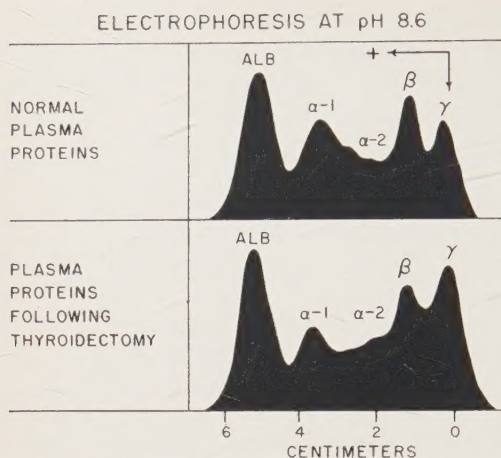


FIG. 1. Effect of thyroidectomy on plasma proteins of the rat.

be accounted for almost entirely by a significant increase in the hexosamine content of both the beta and gamma globulin fractions. The albumin hexosamine was also increased but this contributed little toward the total plasma hexosamine change. The alpha globulins were unaffected. With the known normal distribution of plasma proteins and hexosamine in plasma proteins (Table I), the concentration of hexosamine in each fraction can be estimated (assume a normal plasma protein concentration of 6%). As can be seen in the lower part of Table I, the alpha globulins were the richest hexosamine-containing proteins with albumin and gamma globulin having the lowest concentrations.

Discussion. Normal rat plasma contains all the major protein components seen in human plasma. Whereas human plasma normally contains more alpha-2 globulin, the reverse appears to be true in rat plasma. Many workers have reported difficulty or inability to demonstrate alpha globulins in normal rat serum with the use of moving boundary electrophoresis(8-13). Experience in our laboratory has shown that the separation of the alpha globulins by zone electrophoresis, although much slower with rat than with human plasma, will result with more time or higher voltage. It is likely that the reported absence of alpha globulins by Moore *et al.*(8,9) was in actuality a failure to separate albumin from alpha globulins. That rat plasma normally

contains alpha globulins has clearly been demonstrated here and elsewhere(12).

The distribution of hexosamine in normal rat plasma proteins is similar to that seen in human serum(2), being richest in the alpha globulins(14,15).

The plasma mucoprotein fraction described by Winzler(16) is rich in hexosamine and migrates electrophoretically at pH 8.4 with alpha globulins(17). Mancini, Garberi, and de la Balze(18) have reported that in severe myxedema the mucoproteins, as determined chemically by Winzler's(16) method, are increased. On the other hand, Lewis and McCullagh(19) reported that the alpha-globulins are increased in hyperthyroidism and decreased in hypothyroidism. In support of this is the observation that plasma mucoproteins, as measured electrophoretically at pH 4.5, are increased in hyperthyroidism and decreased in hypothyroidism(20). These divergent observations are difficult to resolve without further study.

In the rat the alpha globulins do not appear to be altered following thyroidectomy. This is contrary to the observations of Moore, Levin, Smelser(9), who found that alpha globulins were absent in normal rats but appeared following thyroidectomy (see above), but is in agreement with the earlier findings of Levin and Leatham(3) that serum globulins in the rat, as measured chemically, increase following thyroidectomy. The findings here indicate that the greatest protein increase is in the gamma globulin fraction and that both the beta and gamma globulin fractions account for the increase in plasma hexosamine seen following thyroidectomy(1).

Since thyroidectomized rats do not develop the alpha-globulin changes which are seen in humans with hypothyroidism this probably represents a species difference.

Plasma fibrinogen is closely associated with, but poorly separated from, beta globulins(11) in the rat. It could not be clearly distinguished in the studies reported here.

Summary. Following thyroidectomy the rat develops a relative increase in gamma globulin. The increase in total plasma hexosamine levels following thyroidectomy can be

almost entirely accounted for by the increase in the hexosamine content of the beta and gamma globulin fractions. The normal distribution of hexosamine in rat plasma is as follows: Albumin 5%, alpha globulins 67%, beta globulins 19%, and gamma globulin 9%. The richest fraction is alpha-1 globulin which contains approximately 4.4% hexosamine. The other globulins contain less and albumin contains only 0.30%.

1. Boas, N. F., and Foley, J. B., *Endocrinology*, 1955, v56, 305.
2. Boas, N. F., and Reiner, M., *J. Clin. Endocrinol.*, 1951, v11, 890.
3. Levin, L., and Leatham, J. H., *Am. J. Physiol.*, 1942, v136, 306.
4. Boas, N. F., and Peterman, A. F., *PROC. SOC. EXP. BIOL. AND MED.*, 1953, v82, 19.
5. Boas, N. F., *J. Biol. Chem.*, 1953, v204, 553.
6. Durrum, E. L., *J. Am. Chem. Soc.*, 1950, v72, 2943.
7. Kunkel, H. G., and Tiselius, A., *J. Gen. Phys.*, 1951, v35, 89.
8. Moore, D. H., Levin, L., and Leatham, J. H., *J. Biol. Chem.*, 1944, v153, 349.

9. Moore, D. H., Levin, L., and Smelser, G. K., *ibid.*, 1945, v157, 723.
10. Li, C. H., and Reinhardt, W. O., *ibid.*, 1947, v167, 487.
11. Deutsch, H. F., and Goodloe, M. B., *ibid.*, 1946, v161, 1.
12. Gjessing, E. C., and Chanutin, A., *ibid.*, 1947, v169, 657.
13. Jameson, E., and Alvarez-Tostado, C., *PROC. SOC. EXP. BIOL. AND MED.*, 1939, v40, 476.
14. Weimer, H. E., Mehl, J. W., and Winzler, R. J., *J. Biol. Chem.*, 1950, v185, 561.
15. Werner, I., and Odin, L., *Uppsala Läkar, Förh.*, 1949, v54, 69.
16. Winzler, R. J., Devor, A. W., Mehl, J. W., and Smyth, I. M., *J. Clin. Invest.*, 1948, v27, 609.
17. Mehl, J. W., Golden, F., and Winzler, R. J., *PROC. SOC. EXP. BIOL. AND MED.*, 1949, v72, 110.
18. Mancini, R. E., Garberi, J. C., and de la Balze, F. A., *Rev. Soc. Argent. de Biol.*, 1951, v27, 285.
19. Lewis, L. A., and McCullagh, E. P., *Am. J. Med. Sc.*, 1944, v208, 727.
20. Mustacchi, P., Peterman, M. L., and Rall, J. E., *J. Clin. Endocrinol. and Metab.*, 1954, v14, 729.

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Stimulation of Adenine Uptake into Mouse Lung and Liver *in vitro* By Tumor Extracts.* (21923)

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Kelly and Jones(1) observed that repeated injections of tissue mashings into normal animals bring about changes in the nucleic acid turnover of the spleen and liver. Our studies (2) have indicated that single injections of a tumor homogenate into normal mice alter the incorporation of adenine-8-carbon¹⁴ (C¹⁴) into the deoxyribonucleic acid (DNA) of

several tissues. Fractions obtained from tumor homogenates by centrifugation or pH adjustment were tested for the presence of the factor(s) responsible for this effect by injection into normal mice simultaneously with adenine-8-C¹⁴ solution. Increase of lung DNA C¹⁴ activity in animals which had received the tumor preparations was taken as an indication that the factor(s) was present in the preparation. Goldwasser(3) reported that an *in vitro* system consisting of rat liver slices or of cell-free homogenates of pigeon liver incorporated adenine into ribonucleic acid but not into DNA. In simplifying the assay procedure for the tumor factor(s), the authors have developed an *in vitro* system in which significant increases in DNA C¹⁴ radioactivity

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after proper incubation with adenine-8-C¹⁴ have been observed.

Methods. The *in vitro* system consisted of a tissue mince prepared from the lungs and livers of normal 9- to 11-week-old C57 black mice.[§] The lungs and livers were rapidly excised, weighed, placed on a watch glass, and minced together by scissors and scalpel until a finely divided mince of the combined tissues was obtained. Approximately equal portions of the mince were placed in wide mouth, 4-inch centrifuge tubes. Three to 5 ml of Ringer's solution fortified with glucose and buffered to pH 7.4 with a .01 ionic strength phosphate buffer was added to each tube. The tubes were maintained at 37°C in a water bath, and the contents of the tubes were continuously aerated by forcing air through long capillaries opening near the bottom of the solution in each tube. The same amount of adenine-8-C¹⁴ was then added to each tube, approximately 0.5 μ c. Tumor homogenate of lyophilized tumor was added to the assay tubes. Other tubes, used as controls, were heated in boiling water for 5 minutes. Following a 4-hour incubation period, the contents of each tube were transferred to narrow centrifuge tubes, and the solid matter was spun down. The supernate was discarded, and the solid matter was washed twice in 10 ml of distilled water; the washings were also discarded. The remaining solids were weighed, transferred to Ten Broeck homogenizers, and homogenized in distilled water to 15 ml total volume. Five ml of the homogenate were taken for DNA extraction by the procedure of Schmidt and Thannhauser(4); other fractions extracted by this procedure were discarded. The DNA was dried on aluminum discs, and C¹⁴ radioactivity was determined by employing an automatic, windowless, gas-flow counter developed in this laboratory(5). Values of phosphorus concentration of the DNA were obtained utilizing the colorimetric method of King(6). Adenine-8-C¹⁴ was synthesized by the procedure of Clark and Kalckar(7). The tumor tissue added to assay tubes was obtained from transplantable mammary carci-

noma E-0771. The tumor was palpable in 10 days and lethal in 30. Tumors were removed approximately 20 days after subcutaneous inoculation and were homogenized in Ten Broeck homogenizers.

To confirm these results, a series of experiments was carried out on liver and also on lung minces. Normal 5- to 10-week-old BAF¹ mice were used and mammary carcinoma E-0771 obtained from BAF¹ tumor bearing mice.[§] The technic used was essentially the same as described above except for the following: exactly 4 ml of Ringer's glucose in phosphate buffer (pH 7.4, ionic strength .01) was used in each tube; stainless steel planchets were used for counting on an automatic Tracerlab Superscaler; and the Adenine-8-C¹⁴ used was obtained from Isotopes Specialties Co. The specific activity of the preparation was 1.93 mc/mM.

Results. The results are expressed in units of disintegrations/second/gram wet weight of washed minced tissue. Values are also calculated in terms of disintegrations/sec./mg DNA phosphorus, and no significant differences between the 2 expressions were found. Duplicate values for each treatment were prepared where 2 values are given.

Groups 5, 6, and 7 were carried out under slightly different conditions and the 2 experiments should not be directly compared, but it is noteworthy that Group 5 falls within the range determined in Group 1.

That *in vitro* incorporation of adenine ac-

TABLE I. Values of Radioactivity for DNA Extracted after Incubation with Adenine-8-C¹⁴.

Group	Treatment of tissue mince	Dis/sec/g wet wt of washed mince tissue
1.	Untreated	4.8, 7.5
2.	Tissue mince heated 5 min. in boiling water	.9, 1.2
3.	Tissue mince + 100-200 mg homogenized tumor tissue	8.3
4.	Tissue mince + lyophilized tumor homogenate corresponding to 400 mg tumor tissue	6.0, 8.9
5.	Untreated	5.5
6.	Tissue mince + 25 mg tumor homogenate	18.4
7.	Tissue mince + 50 mg tumor homogenate	16.2

[§] C57 bl and BAF¹ mice obtained from the Roscoe B. Jackson Memorial Laboratories, Bar Harbor, Me.

TABLE II. Values of Radioactivity for DNA Extracted after Incubation with Adenine-8-C¹⁴.

Group	Treatment of liver mince	Dis/sec/g wet wt of washed mince tissue
8.	Untreated	0
	Liver mince heated 5 min. in boiling water	0
	Liver mince + 25 mg homogenized tumor tissue	3.5
9.	Untreated	0
	Liver mince heated 5 min. in boiling water	0
	Liver mince + 25 mg homogenized tumor tissue (5 Mc adenine-8-C ¹⁴)	25.4
10.	Untreated	0
	Liver mince heated 5 min. in boiling water	0
	Liver mince + 25 mg homogenized tumor tissue	3.7
11.	Untreated	0
	Liver mince heated 5 min. in boiling water	0
	Liver mince + 25 mg homogenized tumor tissue	17.6

tually does occur is indicated by the decreased radioactivity in the tubes where the mince was deactivated by heat. The stimulating effect of tumor tissue on the uptake of adenine into DNA appeared variable, but gives further evidence that active uptake mechanism was operating *in vitro*. It was found in the earlier work referred to above (2) that stimulation of adenine uptake into DNA *in vivo* was also variable. In general, the variability was thought to be due to the lability of the factor(s) in solutions of tumor homogenate.

The results of Group 4, Table I, might indicate that some of the active principle in tumor homogenate can be preserved by lyophilization. The results of Groups 5 and 7 suggest that there may be a limit to the capacity to stimulate adenine uptake. In addition, they indicate that the increased radioactivity subsequent to the addition of tumor homogenate is not merely the effect of the added "active" DNA of the tumor cells, since, in that case, Group 7 should be much higher in value than Group 6.

It was not felt necessary to maintain strictly sterile conditions during the preparation and incubation of the tissue mince since the short

incubation time was not adequate for extensive growth of microorganisms. Microbiological destruction or incorporation of adenine-8-C¹⁴ would have had to have varied from tube to tube to have seriously affected the results.

The results shown on Table II indicate that the same increase in the uptake of labeled adenine is obtained when tumor homogenate is added to a liver mince. This was verified in 4 studies. In no instance was this effect noted in lung minces. A final control study was carried out to ascertain the amount of uptake of adenine by tumor tissue. A tumor mince was prepared under the same conditions as the untreated and the heated controls as described above. The uptake by 25 mg of tumor mince was calculated and found to be 0.5 d/sec. while the heated tumor mince showed no uptake. Thus, the uptake by the amount of tumor added to the liver mince is only a small percentage of the total obtained for the liver plus the tumor, and we may assume that the radioactivity recorded represents uptake by the liver DNA fraction.

Summary. C57 bl mouse lung-liver mince and BAF¹ mouse liver mince incubated with adenine-8-C¹⁴ showed greater DNA activity than a similar system which had been deactivated by heat. This increased activity was not found with lung mince. A factor(s) present in tumor homogenate and possibly also in lyophilized tumor homogenate increased the DNA radioactivity to values significantly higher than those from tissues incubated without tumor homogenates.

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1. Kelly, L. S., and Jones, H. B., *Am. J. Physiol.*, 1953, v172, 575.
2. Furlong, N. B., Jr., Watson, E. J. P., and Davis, W. E., and Griffin, A. C., in preparation.
3. Goldwasser, E., *J. Biol. Chem.*, 1953, v202, 751.
4. Schmidt, G., and Thannhauser, J. S., *ibid.*, 1945, v161, 83.
5. Nye, W. N., and Teresi, J. D., *Anal. Chem.*, 1951, v23, 643.
6. King, E. J., *Biochem. J.*, 1932, v26, 292.
7. Clark, V. N., and Kalckar, H. N., *J. Chem. Soc.*, 1950, 1029.

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Renal Clearance of Para-Amino Salicylic Acid in the Dog.* (21924)

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(Introduced by Eric Ogden.)

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In comparing the renal clearances of para-amino salicylic acid (PAS) and inulin in man, Horne and Wilson(1) found that the former compound had a higher clearance than the latter: The excess clearance was depressed below the level of the inulin clearance and the plasma level of PAS elevated by the administration of caronamide. On the basis of these findings, it has been postulated that there is renal tubular excretion of PAS and a number of reports(2-4) have indicated that the administration of tubular blocking agents (caronamide and Benemid) will raise the level of plasma PAS in man. However, others(5-7) have failed to show persistent differences in plasma PAS levels after oral doses when Benemid is employed as a tubular blocking agent. In the present study we have investigated the renal clearance of PAS in the dog. Our results indicate that the clearance of PAS in this species is less than the glomerular filtration rate. We have furthermore found that Benemid does not depress the renal clearance of PAS.

Methods. Female dogs anesthetized with sodium pentobarbital (30 mg/kg intravenously) were used in all experiments. In prolonged PAS infusion experiments, supplementary anesthesia was given by including the barbiturate in the infusion in quantity sufficient to supply 3 mg/kg/hour.

For the determination of bladder clearances, a priming dose of 1.4-1.6 g each of sodium PAS (Parke-Davis Co.) and creatinine in 50 ml saline was followed by an infusion through a constant infusion syringe pump of the same solution at .5 ml/min. Adequate urine flow was secured by the administration of 20 ml of 10% sodium chloride solution. After a 10-30 minute period of equilibration, the bladder was washed with three 50 ml volumes of distilled water and the wash-

ings were analyzed for both PAS and creatinine. The clearance periods were then begun. Three consecutive clearance periods of 20 minutes each were run, arterial blood samples being taken at the beginning and end of each period. Aliquots of plasma and dilute urine were precipitated with cadmium hydroxide. PAS was determined on the supernate by the method of Newhouse and Klyne(8), and creatinine by the method of Bonsnes and Taussky(9). The color measurements were carried out on a Beckman DU spectrophotometer at 545 mu for creatinine. The recovery of each substance was estimated by adding a standard amount of both to blank plasma and comparing the color developed with that of the standard alone. Recovery of added PAS varied from dog to dog ranging from 63 to 99%. Duplicate determinations on any one sample, however, checked within 5%. Variable recovery of added PAS from plasma has been previously reported(8) but remains unexplained. Plasma concentrations were corrected for the PAS and creatinine recoveries. In the calculation of the bladder clearance of PAS, the plasma concentration was corrected for PAS binding. The original plasma concentration was multiplied by the ratio between ultrafilterable and total plasma PAS as determined on a separate blood sample for each dog. Plasma ultrafiltrates were made by subjecting whole blood in 1/4 inch cellophane tubing to 15 lb air pressure. The ultrafiltrate was collected in two separate portions which were analyzed separately. The PAS concentrations of the ultrafiltrate did not change with time within the limits of the analytical method. In all bladder clearance experiments, the creatinine clearance was simultaneously measured. The plasma creatinine level was corrected for the small amount of creatinine binding by the use of the ultrafiltrate/plasma creatinine factor.

The volumes of distribution of PAS and

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TABLE I. Renal Clearances and Volume of Distribution of PAS and Creatinine in the Dog (9 Animals).

Dog #	Wt, kg	Cl_{cr} , ml/kg/min.	Cl_{PAS} , ml/kg/min.	Cl_{PAS}	V_{cr}^* % B.W.	V_{PAS} % B.W.	V_{PAS}
				Cl_{cr}			V_{cr}
1	9.6	4.2	3.1	.75			
2	9.5	3.9	3.4	.86			
3	11.4	3.3	2.9	.87			
4	20.0	4.8	3.7	.77			
5	18.0	4.1	3.8	.93			
6	15.4	2.1	1.6	.75	45	41	.90
7	13.0	3.7	3.5	.94	43	46	1.07
8	14.9	4.2	3.4	.82	44	44	1.00
9	15.5	5.0	2.9	.58	53	52	.99

* Volumes determined by the method of Greenberg *et al.*(10).

creatinine were estimated by the method of Greenberg *et al.*(10). From the total amount of PAS and creatinine administered in the primer and infusion, was subtracted the amount found in the bladder at the time that the first plasma sample was taken. The difference, representing the total amount of PAS and creatinine in the body was divided by the equilibrium concentration of the 2 substances in unbound form. No attempt was made to estimate the content of either substance in the renal dead space, so that these estimates of volumes of distribution were falsely high(11).

In experiments employing Benemid, the bladder clearance of PAS was determined as described above. After 3 consecutive clearance periods 0.5 g of Benemid dissolved in an equivalent amount of $NaHCO_3$ (12) was injected intravenously in a 50 ml volume. The pH of the solution was 7.8. After a 10-minute period the PAS clearance was again determined in 3 consecutive periods.

Results. Binding of PAS by plasma proteins. In 12 measurements of the ratio of PAS concentration in the ultrafiltrate to that in the plasma, the free PAS was found to account for 49 to 88% of the total. The average value was 67% and the standard deviation was $\pm 9\%$. This variability in the protein binding has been noted previously in the case of other salicylates(13) and the binding of PAS in human blood is likewise variable (14).

Bladder clearance of PAS. In the first 5 bladder clearance experiments, the clearance was calculated from the plasma PAS concentration corrected by the average value for pro-

tein binding. In the last 4 experiments, the actual PAS binding by the individual dog's plasma was used in the calculation. The PAS clearance values ranged from 1.5-3.8 ml/kg/min. The creatinine clearances ranged from 2.0-5.0 ml/kg/min. The ratio of PAS to creatinine clearance ranged from .58 to .94 with an average of .81. In only 1 of 37 clearance periods did the ratio exceed 1. In this period the clearance ratio was 1.1.

Effect of Benemid on the bladder clearance of PAS. The bladder clearances of PAS in the 3 Benemid experiments were 20.8, 22.5, and 47.0 ml/min before the administration of Benemid and 30.7, 29.6 and 47.0 ml/min respectively afterwards. The creatinine clearance was unchanged in the first 2 experiments and rose slightly (from 65 to 76 ml/min) in the third.

Volumes of distribution of Creatinine and PAS. The volume of distribution calculated from the equilibrium plasma concentration and the PAS balance is, as has been noted, falsely high because of the PAS contained in the renal dead space. The average value obtained in experiments in which this method was employed was 45.5% of the body weight. In the same experiments, the volume of distribution of creatinine similarly calculated (and with almost the same error) was 46% of the body weight. We have noted elsewhere (11) that the true volume of distribution of creatinine as determined by disappearance curve analysis which eliminates the dead space error is closer to 35% of the body weight. Similar analyses of disappearance curves after single injections of PAS which

are not reported here yielded almost identical values for the volume of distribution of this substance (34% of the body weight). A similar value has been reported for the dog by Way *et al.* (14).

Discussion. The results demonstrate that in the dog the clearance of PAS is less than the glomerular filtration rate as measured by the clearance of creatinine. The fact that the clearance ratio is less than one may be interpreted to indicate that tubular reabsorption of PAS occurs in excess of tubular secretion of this molecule, if indeed the latter occurs at all. The possibility that secretion and reabsorption of PAS occur together appears unlikely because of the relatively narrow range of clearance ratios observed between PAS and creatinine. We were further unable to observe the diminution of PAS clearance with Benemid which would have been expected if this material interfered with the tubular secretion of PAS. In fact, in 2 of 3 experiments, Benemid caused an increase in the clearance of PAS.

The evidence for tubular secretion of PAS upon which the rationale of the use of tubular inhibitors is based is scanty. Only Horne and Wilson (1) appear to have compared PAS clearance and filtration rates. Others have confined their studies to the measurement of plasma levels after the oral administration of PAS with or without tubular inhibitors.

The present experiments indicate that in the dog at least, there is no evident tubular excretory activity toward PAS. If, in fact, plasma levels of PAS are elevated by such inhibitors, a finding which has been questioned repeatedly (5-7), it would appear that the site of action is not on the renal tubule.

Summary. Simultaneous measurements of the renal clearance of PAS and creatinine in the dog indicate that the former substance is not excreted by the renal tubules. Benemid does not appear to diminish the renal clearance of PAS in the dog. This raises question as to the rationale of employing Benemid as an adjunct to PAS therapy in man. The volume of distribution of PAS appears to correspond closely with that of creatinine.

1. Horne, N. W., and Wilson, W. M., *Lancet*, 1949, v257, 507.
2. Boger, W. P., and Pitts, F. W., *Science*, 1950, v112, 149.
3. Carr, D. T., Karlson, A. G., and Bridge, E. V., *Proc. Staff Mayo Clinic*, 1952, v27, 209.
4. Boger, W. P., Beatty, J. O., Pitts, F. W., and Flippin, H. F., *Ann. Int. Med.*, 1950, v33, 18.
5. Rieber, C. W., Saline, M., and Freedman, M. M., *Am. Rev. Tuberc.*, 1951, v64, 448.
6. Riley, C., Clowater, R. A., and Shane, S. J., *Diseases of Chest*, 1953, v23, 90.
7. Rekola, J., *Acta Tuberc. Scand.*, 1953, v28, 113 (abstract).
8. Newhouse, J. P., and Klyne, W., *Biochem. J.*, 1949, v44, vii.
9. Bonsnes, R. W., and Tausky, H. H., *J. Biol. Chem.*, 1945, v158, 581.
10. Greenberg, J., Schwartz, I. L., Spinner, M., Silver, L., and Starr, N., *Am. J. Physiol.*, 1952, v168, 86.
11. Sapirstein, L. A., Vidt, D. G., Mandel, M. J., and Hanusek, G., *ibid.*, 1955, v181, 330.
12. Beyer, K. G., Russo, H. F., Tillson, E. K., Miller, A. K., Verwey, W. F., and Gass, S. R., *ibid.*, 1951, v166, 625.
13. Williams, F., and Leonards, J. R., *J. Pharm. Exp. Ther.*, 1948, v93, 401.
14. Way, E. L., Smith, P. K., Howie, D. L., Weiss, R., and Swanson, R., *ibid.*, 1948, v93, 368.

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Tissue Storage in Cadmium Treated Pigs. (21925)

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During an investigation of the ascaricidal activity of cadmium added to the feed of pigs (1,2), cadmium analyses of various tissues were made. The purpose was to determine the extent to which ingested cadmium was retained and to locate the tissues where greatest storage occurred. Although the above authors reported no evidence of toxicity with the dosage of cadmium used, it was considered desirable to see if tissue levels reached concentrations approaching those previously reported in animals showing definite signs of poisoning.

Method. Pigs, varying in weight from 25 to 150 lb, were fed Aska-Rid* in the diet for 72 hours. They were bathed prior to and following treatment and just before autopsy to prevent contamination of tissue samples. They were fed *ad libitum* during the experiment, and no reduction in food intake was noted for the treatment period. With the type ration used for administering cadmium, a 50-lb pig will eat about 2.5 lb per day(2). Thus, the cadmium oxide intake for 3 days would be 510 mg at the 68 mg per lb concentration. The first group received 68 mg cadmium oxide per lb of feed and were sacrificed at intervals of 14 and 30 days following treatment. This is the cadmium concentration recommended for worming pigs when used in a 3-day feeding program. The second group received 91 mg cadmium oxide per lb of feed and were sacrificed at intervals of 1, 3, 7, 14, 60, and 90 days following treatment. The third group received 68 mg cadmium oxide per lb of feed for 3 days. At the end of 6 weeks, they were again treated with feed containing 68 mg cadmium oxide per lb for 3 days. They were sacrificed 30 days after the second cadmium feeding. The fourth group received no cadmium and served as controls. Cadmium determina-

tions were made in duplicate by the dithiozone method of Church(3).

Results. The highest concentration reported (Table) was 1.30 mg of cadmium oxide per 100 g of fresh tissue. This was from the spleen of a pig fed exclusively on 91 mg cadmium oxide per lb feed for 3 days and autopsied one day later.

Cadmium levels in the various tissues drop rapidly with only the kidney and spleen still showing significant increases as long as 14 days after the feeding experiment. Therefore, in subsequent determinations (30, 60, and 90 days after cadmium feeding), only these 2 tissues and the liver were analyzed. The liver was included, as others had reported high levels in this organ(3,4). When 2 feedings are made at a 6-weeks' interval, cadmium storage is greater than on a single feeding, but in no tissue was it as high as 1 mg per 100 g of fresh tissues 30 days after the second feeding.

Most studies reported on cadmium concentration in tissues are following administration by inhalation. Under such circumstances, the highest concentration is usually in the lungs. In fatal or near-fatal doses, this may be as high as 10 mg per g of dried lung tissue in the rat(4). On the other hand, cadmium oxide inhalation experiments in dogs(5) resulted in concentrations of cadmium oxide as high as 13.3 mg per 100 g of fresh kidney tissue. The highest in the liver was 3.9 mg. In these animals, toxicity was not noted nor were definite pathologic changes seen at autopsy. Prodan(6) fed 10 mg of cadmium per day to cats for 2 months. Emesis occurred occasionally during the first few days and ceased. Near the end of the experiment all animals manifested a decreased appetite. Tissue cadmium was 5.62 mg per 100 g of tissue for the liver, 4.65 mg for the kidney, and 1.11 mg for the spleen. Tipton(7) has recently assembled some very revealing data on cadmium con-

* Aska-Rid is the tradename for Pitman-Moore Co. brand of anthelmintic powder containing 1.5% cadmium oxide in an inert diluent.

TABLE I. Distribution of Cadmium in mg %.

Tissue		Untreated pigs	Treated pigs*							Repeat treatment† with 68 mg/lb CdO
			91 mg/lb CdO					68 mg/lb CdO		
			Days following treatment							
			1	3	7	14	60	90	14	
Liver	.05(3)‡	.22	.12	.16	.16	.36(2)	.28(2)	.02(2)	.41(2)	.92(4)
Kidney	.03(3)	1.05	1.04	.53	.41	.23(2)	.16(2)	.38(2)	.27(2)	.65(4)
Spleen	.05(3)	1.30	.84	.91	.35	.46(2)	.24(2)	.05(2)	.53(2)	.92(4)
Blood	.02	.19	.25	.36	.13			.16(3)		
Brain	—	.97	.39	.09	.06			.02(2)		
Heart	—	.59	.25	.07	.00			.04(2)		
Muscle	—	.25	.17	.02	.04			.02(2)		
Stomach tissue	—	.12	.00	.53	.00			.16(2)		
" contents	—	.48	.06	.09	.00			.06(2)		
Ileum	—	1.10	—	—	—			—		
Cecum	—	1.23	.06	—	—			—		
Mid-jejunum	—	.80	.18	.26	.06			.32(2)		
Colon contents	—	—	—	—	—			.02(2)		
Rib bone	—	—	—	.10	.03			.04(2)		
Subcut. fat	.02	—	—	—	—			.11		
Abdominal fat	.02	—	—	—	—			.16		

* All received food containing indicated concentration of CdO for 3 days.

† Same dose repeated 6 wk after first; pigs sacrificed 30 days after second dose.

‡ No. in parentheses indicates No. of pigs from which average obtained. No number in parentheses indicates only tissue from one pig analyzed.

tent of human kidneys obtained at autopsy. On a fresh tissue basis, the average was 3.31 mg of cadmium per 100 g for kidneys and 0.36 mg for liver. There was no known exposure to cadmium in any of these subjects.

Summary. Feeding cadmium oxide-containing food for 3 days at concentrations of 68 mg and 91 mg per lb of food results in cadmium retention in the liver, spleen, and kidneys. The increase is significant when compared to controls but is very low when compared to cadmium levels others have reported by inhalation or feeding. Humans with no known exposure to cadmium have higher cadmium levels in the kidneys than pigs fed cadmium as described above.

1. Bunde, C. A., Blair, H. E., Burch, G. R., and Lee, J. W., *PROC. SOC. EXP. BIOL. AND MED.*, 1954, v87, 547.

2. Burch, G. R., and Blair, H. E., *J. Am. Vet. M. A.*, 1955, v126, 304.

3. Church, F. W., *J. Indust. Hyg. and Toxicol.*, 1947, v29, 34.

4. Barrett, H. M., Irwin, D. A., and Semons, E., *ibid.*, 1947, v29, 279.

5. Princi, F., and Geever, E. F., *Arch. Indust. Hyg. and Occup. Med.*, 1950, v1, 651.

6. Prodan, L., *J. Indust. Hyg. and Toxicol.*, 1932, v14, 174.

7. Tipton, I. H., Foland, W. D., Bobb, F. C., and McCorkle, W. C., personal communication to Dr. C. A. Bunde.

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Synthesis of Glycogen Fractions by Heart Homogenates.* (21926)

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Glycogen synthesis, *in vitro*, has been investigated in a number of tissues. Hastings (1) reported synthesis in liver, Stadie and Zapp (2) in diaphragm, Stadie, Haugaard and Perlmutter (3) in heart and Marsh and Miller (4) in the kidney. Interest in the glycogen fractions was renewed by the work of Bloom, Lewis, Schumpert and Shen (5), who described a method for the isolation of 2 types of glycogen through differential solubility in cold trichloroacetic acid (TCA). Cordier and Dessaux (6), utilizing this technic in an investigation of cardiac glycogen disappearance in rats subjected to anoxia, found that the TCA soluble fraction decreased to a greater extent than the TCA insoluble fraction of glycogen. A pronounced species variation of these 2 forms of cardiac glycogen was reported by Merrick and Meyer (7), who found in studies on dogs exposed to conditions of fulminating anoxia that although the TCA soluble glycogen disappeared rapidly from the heart, there appeared to be a temporary increase of the TCA insoluble fraction. The problem of changes in the cardiac glycogen fractions is worthy of more detailed analysis. The present study deals with the changes of concentration of the cardiac glycogens in rat and dog hearts, which have been homogenized in a medium promoting glycogen synthesis, and exposed to either an atmosphere of oxygen or an atmosphere of nitrogen.

Methods. For each of the 10 runs in each series of analyses, either the pooled ventricles from 6 decapitated male albino rats or a portion of the left ventricle from a dog anesthetized with pentobarbital sodium (32.5 mg/kg) was removed, weighed and then frozen between blocks of dry ice. Enough cold (1°C) fluid medium (Krebs Phosphate Ringer's solution or 5.4% glucose solution) was

measured to make a 10% weight/volume mixture when homogenized with the heart tissue. The ventricles were ground with the fluid medium in a Ten Broeck Tissue Grinder, the temperature being held at approximately 2-3°C during homogenization. A 3 ml sample of the homogenate was taken for the initial glycogen value. The remainder of the homogenate was transferred to Warburg flasks, 3 ml per flask. The flasks were placed in a Warburg bath set for a temperature of 37.5°C, flushed with O₂ or N₂ and allowed to equilibrate for 30 minutes before oxygen consumption was recorded. One flask of each series of 7 was taken from the bath at succeeding time intervals (as indicated in Table I) and the contents removed for glycogen analysis. The final sample was removed 105 minutes after the initial sample. Extraction of the 2 glycogens was accomplished with essentially the procedure outlined by Bloom, *et al.* (5), that is, the TCA soluble fraction was extracted with cold TCA and separated from the precipitate by centrifugation at 1°C. One modification of the procedure, however, consisted of digesting the precipitate from the TCA extraction with 30% KOH to secure the non-extractable glycogen rather than using a duplicate sample and determining total glycogen by KOH digestion. After hydrolysis in sulfuric acid, the isolated glycogen samples were measured quantitatively by the anthrone procedure of Seifter, Dayton, Novic and Muntwyler (8).

Results and discussion. There was a precipitous drop of both glycogen fractions (Table I) which shows that glycolysis rapidly reduces heart glycogen when the organ has been homogenized. In contrast to the findings of Cordier and Dessaux (6) with the heart of anoxic rats (*in vivo*) where the TCA insoluble fraction was relatively stable, both fractions dropped to about 50% of their initial value within 5 minutes. Although no de-

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TABLE I. Glycogen Content of Heart Homogenates as Mean \pm Standard Deviation.

Animal and medium	Glycogen	Time in min. after heart was homogenized					
		0	5	15	22½	30	60
Rat 5.4% glucose 100% oxygen	TCA soluble	131 \pm 37.7	66 \pm 15.6	55 \pm 8.8	49 \pm 9.6	63 \pm 19.3	79 \pm 31.6
	TCA insoluble	140 \pm 44.9	74 \pm 24.9	33 \pm 15.8	23 \pm 10.7	20 \pm 7.4	16 \pm 6.6
	Total	271	140	88	77	83	95
Rat 5.4% glucose 3% oxygen 97% nitrogen	TCA soluble	154 \pm 51.2	—	—	—	51 \pm 22.5	54 \pm 29.9
	TCA insoluble	102 \pm 37.7	—	—	—	19 \pm 6.4	18 \pm 3.7
	Total	256	—	—	—	70	72
Rat Krebs phosphate Ringer's 1% glucose 100% oxygen	TCA soluble	86 \pm 45.0	—	—	—	32 \pm 43.9	53 \pm 41.3
	TCA insoluble	109 \pm 54.7	—	—	—	25 \pm 7.0	25 \pm 11.9
	Total	195	—	—	—	77	78
Dog 5.4% glucose 100% oxygen	TCA soluble	471 \pm 126.8	405 \pm 165.3	244 \pm 95.4	166 \pm 52.7	125 \pm 41.5	55 \pm 14.8
	TCA insoluble	114 \pm 28.6	105 \pm 16.3	90 \pm 16.7	85 \pm 20.1	80 \pm 20.1	40 \pm 16.8
	Total	585	510	334	251	205	95

Each value represents the mean of 10 determinations.

* 90 min. samples.

terminations were made during the first half hour in the series utilizing Krebs Ringer's or low oxygen in 5.4% glucose, the 30-minute values suggest that they follow the same glycolytic pattern. A plateau independent of initial glycogen concentration is reached at 30 minutes. The TCA soluble glycogen, except when synthesis occurs, stabilizes at approximately 50 mg per 100 g of tissue while that part not extracted by cold TCA drops to 20 mg per 100 g of tissue.

This behavior of rat heart glycogen contrasts strongly with the situation in the dog where the TCA insoluble glycogen is found to decrease gradually over the 105 minute period (Table I). The TCA soluble glycogen of the dog heart, although disappearing rapidly, does not reach a stable level until 75 minutes after the first sample is taken.

Synthesis of glycogen by the rat heart was achieved in oxygenated 5.4% glucose confirming the experience of Stadie, Haugaard and Perlmutter(3). The most marked synthesis was in the TCA extractable portion, which after declining to 54 mg per 100 g of tissue, then began to rise and continued until a value of 103 mg per 100 g of tissue was reached at the end of 105 minutes. This represents synthesis at the rate of 0.594 mg of glycogen per 100 g of heart tissue per minute. The TCA insoluble glycogen, however, continued to decline for 75 minutes and then rose from 16 to 22 mg per 100 g of heart tissue at 105 minutes, a difference significant to the 98% confidence level. The insoluble fraction is resynthesized at roughly one-third the rate that the TCA soluble glycogen is formed.

Nothing in the data from rat heart homogenates suggests that the TCA soluble glycogen undergoes glycolysis in preference to the TCA insoluble fraction, although this is the implication from the work of Cordier and Dessaux(6) on the intact animal. However, this does seem to be true of the homogenized dog heart. The homogenization of the tissue may well alter metabolic pathways by destroying the normal anatomy of the cell.

The resynthesis of glycogen, under the conditions of the experiment, starts with the formation of the TCA soluble moiety. Whether or not the TCA insoluble fraction is

formed directly from the glucose, or represents the binding of the soluble form to proteins or some other molecule which is insoluble in cold TCA has yet to be determined.

The dog heart homogenate did not synthesize either glycogen fraction under the same conditions in which the rat heart showed glycogenic activity. Neither form of glycogen of rat heart was synthesized in Krebs Ringer's, though adequate glucose was present, nor in 5.4% glucose when oxygen tension was reduced to 21.5 mm Hg.

Glycogen synthesis was well correlated with oxygen consumption of the tissue homogenates. In the one medium (oxygenated 5.4% glucose) which promoted glycogenesis, oxygen consumption was 303 $\mu\text{l-O}_2$ per g of wet tissue per hour compared to the utilization of 145 $\mu\text{l-O}_2$ per g of wet tissue per hour in the unoxygenated sugar medium and the 131 $\mu\text{l-O}_2$ per g of wet tissue per hour in the oxygenated Krebs Ringer's. The dog heart suspension exhibited the lowest oxygen uptake, using only 114 $\mu\text{l-O}_2$ per g of wet tissue per hour.

Summary. Glycolysis reduces both glycogen fractions of the rat heart at about the same rate for the first 5 minutes, regardless of the medium used for suspension of the tis-

sue. In Krebs Phosphate Ringer's solution and in unoxygenated 5.4% glucose, the 2 glycogens drop to a low, but stable level. The glycogen synthesized in the sugar medium is primarily the TCA soluble glycogen. The TCA soluble glycogen begins synthesis at the end of 22.5 minutes and synthesis of the TCA insoluble fraction begins 50 minutes later. A distinct species difference was observed in the metabolism of cardiac glycogen in the rat and dog, inasmuch as the TCA insoluble fraction was relatively more stable in the dog.

1. Hastings, A. B., *Harvey Lectures*, 1940-41, v36, 91.
2. Stadie, W. C., and Zapp, J. A., *J. Biol. Chem.*, 1947, v170, 55.
3. Stadie, W. C., Haugaard, N., and Perlmutter, M., *ibid.*, 1947, v171, 419.
4. Marsh, J. B., and Miller, K. L., *Science*, 1953, v118, 416.
5. Bloom, W. L., Lewis, G. T., Schumpert, M. Z., and Shen, T., *J. Biol. Chem.*, 1951, v188, 631.
6. Cordier, D., and Dessaux, G., *J. Physiologie*, 1952, v44, 703.
7. Merrick, A. W., and Meyer, D. K., *Am. J. Physiol.*, 1954, v177, 441.
8. Seifter, S., Dayton, S., Novic, B., and Muntwyler, E., *Arch. Biochem.*, 1950, v25, 191.

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Clotting Factor X. Physiologic and Physico-Chemical Properties. (21927)

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The use of serum from various pathological conditions in the thromboplastin generation test(1,2) led to the conclusion that besides the serum factors already known (Factor VII = SPCA, proconvertin and Factor IX = Christmas factor, PTC)* another clotting factor exists in serum for which the designa-

tion Factor X(3-5) was proposed. Matching experiments with serum from hemophilia B (deficient in Factor IX) proved to be especially demonstrative.

Method. A modification of the thromboplastin generation test developed in our laboratory was employed(2). The 4 components incubated to generate blood thromboplastin are: Platelet suspension prepared according to Flückiger *et al.*(6), calcium chloride, oxalated plasma adsorbed on barium sulfate (source of antihemophilic globulin) and serum. The first 3 components are kept

*The following numbering of clotting factors is proposed: Fibrinogen (F.I), Prothrombin (F.II), Thromboplastin (F.III), Calcium (F.IV), Labile factor, Proaccelerin (F.V & VI), SPCA Proconvertin (F.VII), antihemophilic Globulin (F.VIII), P.T.C., Christmas Factor (F.IX).

constant (normal platelets and oxalated plasma being used), whereas serum is varied. The results of the test are given in incubation and clotting-times directly measured (not in thromboplastin-units). To study the mixture of 2 sera, both are diluted 1/10 with buffer, then 0.15 cc of each dilution added to the mixture of platelet suspension, adsorbed plasma and CaCl_2 . *Quantitative determination of Factor VII*: according to Koller, Loe-liger and Duckert(7). *Chloroform extract of human brain thromboplastin*. Acetone dried brain powder (1 g) is suspended in chloroform at room temp. 20 min, then filtered and washed with chloroform, (final vol. 50 cc). After evaporation of solvent at 50°C the residue is suspended in 50 cc physiological saline and stored at 30°C . This stock solution is diluted 1/100 with veronal-acetate buffer before use in the thromboplastin generation test, replacing the thrombocytes.

Purification of Factor IX and X. All operations at room temperature. a). *Purification of Factor IX by chromatography*. To prepare the column, 1 g of barium sulfate and 1 g of hyflosupercel are suspended in physiological saline, pH 7, and introduced into the column. Excess saline is discarded. Normal citrated serum (1.8 cc serum + 0.2 cc trisodium citrate 0.2 M) is poured on top of column. After the serum 0.5 cc saline is added and elution performed by means of trisodium citrate, 0.14 M, pH 7.8. Flowing speed is slow (1 drop/25 sec.). The eluate is fractionated into portions of 1 cc. The first fractions are rich in Factor VII. Factor IX appears in the fourth fraction. By chromatography Factor IX is obtained free of Factor X which is not eluted by this method. The fraction contains however 5 to 35% of Factor VII. b). *Purification of Factor IX by adsorption*. 10 cc of old normal serum (stored at room temperature a few days in order to remove Factor X) and 0.8 cc trisodium citrate 0.2 M (final concentration 0.0148 M) are mixed with 600 mg barium sulfate and stirred 5 min. with a glass rod. After centrifugation, the barium sulfate is washed with 2 cc distilled water and the supernatant discarded. Elution: the barium sulfate is suspended in 1 cc of trisodium citrate 0.14 M,

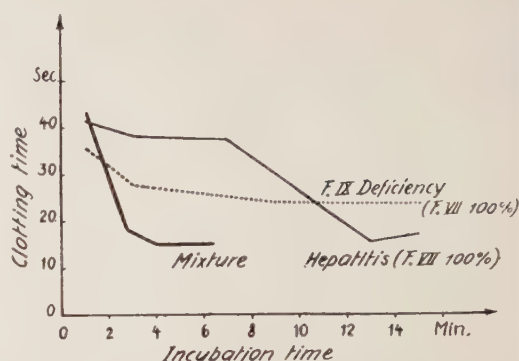


FIG. 1. Normalisation of thromboplastin formation with a mixture of hemophilia B-Serum (F IX-deficiency) and hepatitis-serum.

pH 7.8, stirred 5 min. and centrifuged. The supernatant solution contains Factor IX, traces of Factor VII and is Factor X-free. c). *Purification of Factor X by adsorption*. 4.5 cc normal serum + 0.5 cc trisodium citrate 0.2 M (final concentration 0.02 M) are mixed with 250 mg barium sulfate and stirred 5 min. After centrifugation the supernatant is discarded, barium sulfate washed with 2 cc distilled water and the solution discarded. Elution: barium sulfate is suspended in 1 cc of trisodium citrate 0.14 M and stirred 3 min. The solution contains Factor X, traces of Factor VII and is Factor IX-free. To exclude any contamination with Factor IX this preparation may be made with hemophilia B-serum.

Results. Two types of abnormal thromboplastin generation curves. (Fig. 1) Normal serum yields a curve with minimal clotting time of 11 to 12 seconds after incubation time of 3 to 8 minutes. Serum from hemophilia B (deficient in Factor IX) gives a markedly prolonged minimal clotting time, reached however after a normal or almost normal incubation time. On the other hand serum from a patient treated with 3-(1-phenyl-propyl)-4-hydroxycoumarin (= Marcoumar), or from a patient with hepatitis (Fig. 1) produces a greatly delayed curve (incubation time over 12 minutes), but the clotting time finally reached is within normal limits. This curve corresponds to Factor X-deficiency. The difference in shape of the curve is even more pronounced when instead of platelet suspension a

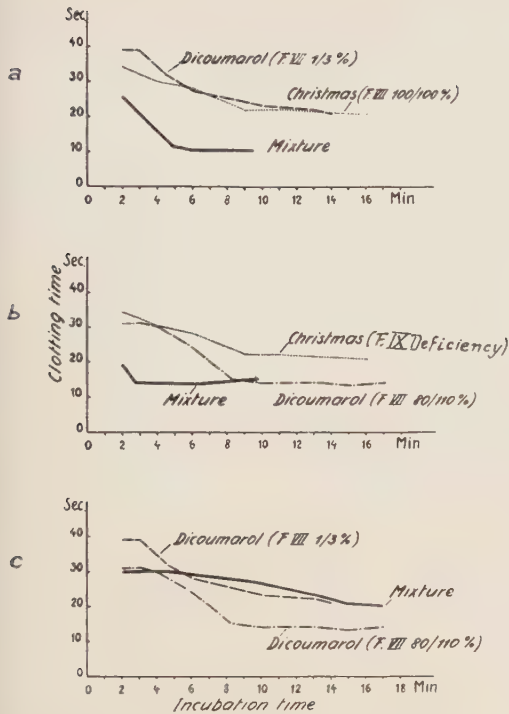


FIG. 2. Effect of a dicoumarol derivative (Marcoumar) on thromboplastin formation. (Factor VII activity is given in plasma and serum.) In Exp. C, the concentration of Factor IX is even higher in the mixture than in assay a and b (in Christmas-serum Factor IX is very low), and the concentration of Factor VII is the same as in assay a. Nevertheless thromboplastin formation of the mixture is very poor in c but normal in a and b. A 3rd serum factor has to be assumed.

chloroform extract of brain is used in the generation test. If the concentration of Factor X is very low, thromboplastin formation is almost impossible. Therefore the clotting time (reciprocal to amount of thromboplastin formed) remains high as in Factor IX-deficiency, in these extreme cases(13).

Differentiation of Factor X from Factor VII and IX by matching experiments. A mixture of equal parts of the above mentioned Marcoumar-serum and hemophilia B-serum yields an entirely normal thromboplastin generation. (Fig. 2a). This finding might be in agreement with the hypothesis that insufficient thromboplastin generation of Marcoumar-serum is due to Factor VII-deficiency while that of hemophilia B-serum is caused by lack of Factor IX. However the following observation contradicts this view: Serum of a

patient who had first been given Marcoumar, then, because of bleeding, vit. K₁, and whose Factor VII concentration had returned to normal (100% in plasma and in serum) produced a markedly delayed thromboplastin generation. Hemophilia B-serum normalises this clotting defect. (Fig. 2b). Therefore lack of Factor IX cannot account for the delay of thromboplastin generation, nor can Factor VII-deficiency do so. A third serum-factor (Factor X) must be involved. If the 2 Marcoumar sera (with 10 resp. 100% Factor VII) are mixed, there is no normalisation of thromboplastin generation. (Fig. 2c) Serum of epidemic hepatitis, of hepatic cirrhosis, of the new-born behaves in the same way as Marcoumar-serum. (Table I). Stored serum is also abnormal in thromboplastin generation although the concentration of Factor VII and IX remain unchanged during several days at room temperature. After 8 days of storage Factor VII 160% and Factor IX were normal. The delayed thromboplastin formation is therefore due to deficiency of another serum factor.

Purified Factor IX, mixed with hemophilia B-serum produces a normal thromboplastin generation; the same is true with purified Factor X when mixed with Marcoumar-serum. Matching of both purified preparations (Factor IX and X) gives a normal thromboplastin generation in spite of the very low Factor VII content of the mixture. The chromatographic fraction containing no factor X produces a very delayed thromboplastin generation when mixed with Marcoumar-serum.

Objections to these matching experiments. Hemophilia B-serum used in most matching experiments (Table I) contains considerable amounts of prothrombin, as a consequence of the reduced prothrombin consumption. During the thromboplastin generation test prothrombin is converted into thrombin and therefore may influence the results. Isenschmid(9), using fibrinogen as substrate instead of calcium-free plasma, was able to measure the amount of thrombin formed simultaneously with thromboplastin generation. This amount was subtracted from the total quantity of thrombin measured with calcium-free plasma as substrate. In this way a "cor-

TABLE I. Matching Experiments.*

No.	Serum I					Serum II				Mixture†				Thromboplastin generation	
	F.	VII	IX	X		F.	VII	IX	X	F.	VII	IX	X		
	%					%				%					
1. Marcoumar	3	+	—		Hemophilia B	100	—	+		50	+	+		normal	Fig. 2a
2. Marcoumar + vit. K1	110	+	—		"	100	—	+		105	+	+		"	Fig. 2b
3. Hepatitis, mild	100	+	—		"	100	—	+		100	+	+		"	Fig. 3
4. <i>Idem</i>	100	+	—		Marcoumar	40	+	—		70	+	—		delayed	
5. Hepatic cirrhosis	75	+	—		Hemophilia B	100	—	+		90	+	+		normal	
6. New-born	45	+	—		"	100	—	+		75	+	+		"	
7. Stored normal serum (8 days)	160	+	—		"	100	—	+		130	+	+		"	
8. Factor IX purif. chromat.	35	+	—		"	100	—	+		70	+	+		"	
9. <i>Idem</i>	35	+	—		Marcoumar	25	+	—		30	+	—		delayed	
10. Factor IX purif. adsorpt.	7	+	—		Hemophilia B	100	—	+		55	+	+		normal	
11. <i>Idem</i>	7	+	—		Marcoumar	30	+	—		20	+	—		delayed	
12. Factor X purif. adsorpt.	7	—	+		"	35	+	—		20	+	+		normal	
13. Factor X purif.	7	—	+		Hemophilia B	100	—	+		55	—	+		abnormal	
14. Factor X purif. adsorpt.	7	—	+		Factor IX purif. adsorpt.	7	+	—		7	+	+		normal	

* All sera I and II used alone, produce an abnormal thromboplastin generation; Factor IX and X can be determined only approximately; + means normal, — means diminished or lacking activity in thromboplastin generation test; "purif. chromat." means purified by chromatography; "purif. adsorpt." means purified by adsorption.

† In a mixture of 2 sera resulting in normalisation of thromboplastin generation Factor IX and X concentrations do not exceed 50% of normal values.

As example of normalisation see Fig. 1, 2a and b.

As example of non-normalisation see Fig. 2c.

rected" curve was obtained, which showed that normalisation of thromboplastin generation, by mixing Marcoumar- and hemophilia B-serum, persists. Only the "over-normalisation" has to be attributed to the prothrombin content of hemophilia B-serum. Isenschmid could demonstrate that thrombin has no accelerating effect on thromboplastin formation; therefore simple subtraction of its effect is allowed. Another objection is furnished by the observation that matching of Marcoumar- and hemophilia B-serum does not always produce a complete normalisation of thromboplastin generation. In fact Dicoumarol and its derivatives although they lower primarily Factor X (besides prothrombin and Factor VII), affect also, to a lesser degree, Factor IX. Marcoumar, (the Dicoumarol-derivative we used most), lowers first the concentration of Factor X alone, and after a week or 2 the activity of Factor IX. Bis-3, 3'-(4-hydroxycoumarinyl) - ethyl - acetate (tro-

mexan), on the other hand, seems to lower Factor IX more rapidly. It is evident that hemophilia B-serum cannot normalise the clotting defect of a serum simultaneously deficient in Factor IX and X. It has been suggested that a factor-X deficiency is a slight deficiency of Factor IX. This opinion is in contradiction to the results obtained by dilution of normal serum(2) where both incubation and coagulation time are prolonged, not incubation time alone. The assays with purified factors (Table I, No. 8-14) also contradicts this opinion as well as the experiments illustrated in Fig. 2. Another objection was that an excess of *antihemophilic globulin* might explain normalisation produced by mixing hemophilia B-serum with various pathological sera (Table I). We increased the concentration of antihemophilic globulin in the incubation mixture 5 times (by using a 1:5 instead of a 1:25 dilution of BaSO₄-plasma) without obtaining a normalisation of throm-

boplastin generation with hemophilia B or Marcoumar-serum. It is evident that excess of one clotting factor can compensate *partially* for the deficiency of another factor. A complete normalisation however has never been achieved by this procedure.

Insignificant role of Factor VII in blood thromboplastin formation. In Table I it may be seen (No. 14) that thromboplastin generation of the mixture is normal with as low a Factor VII content as 7% (100% Factor VII = normal concentration in plasma) whereas it is delayed with a concentration of 70% (No. 4 of Table I). A rise of Factor VII from 10 to 110% under the influence of Vit. K does not change significantly the delayed thromboplastin formation (Serum I in No. 1 & 2 of Table I). On the other hand a decrease of Factor VII in serum from 150% to 18% in the beginning of Marcoumar-treatment did not delay thromboplastin formation. It may therefore be concluded that Factor VII has very little, if any, influence in blood thromboplastin generation. (See also Fig. 2).

Physiological properties of Factor X compared with those of Factor IX. Incubation mixtures are prepared with constant amounts of all factors necessary for thromboplastin formation except Factor X, the concentration of which is varied from 1 to 100%. As seen in Fig. 3 the speed of thromboplastin formation is greatly enhanced by increasing amounts of Factor X, whereas the *amount* of thromboplastin produced (*i.e.* the clotting time finally reached) is practically unchanged. If in the incubation mixture the con-

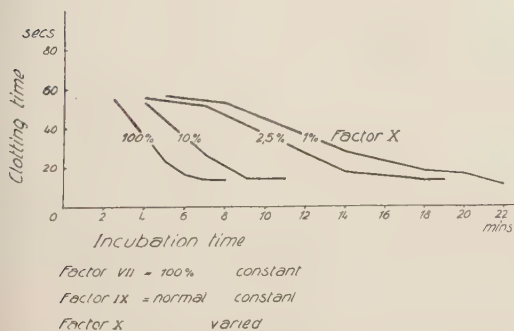


FIG. 3. Influence of variable Factor X concentrations, all other factors being kept constant. Clotting time normal, incubation time prolonged.

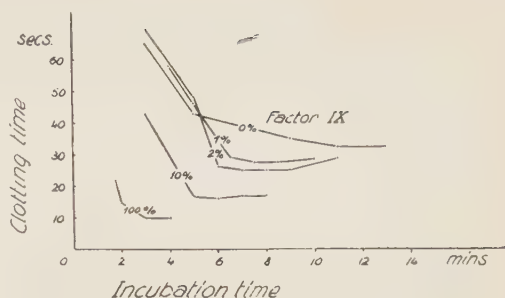


FIG. 4. Influence of variable Factor IX concentrations, all other factors being kept constant. Incubation time in normal range, clotting time prolonged.

centration of Factor IX is varied while all other factors are kept constant, the clotting time (corresponding to amount of thromboplastin formed) is primarily influenced (Fig. 4), whereas incubation time differs only slightly. It seems therefore that Factor IX is a precursor of blood thromboplastin and that Factor X has to be considered as an *accelerator of blood thromboplastin formation*. There is another difference between the 2 factors: in Factor IX-deficiency prothrombin consumption is markedly reduced whereas in "pure" Factor X-deficiency it is normal.

Physico-chemical properties of Factor X. The properties of Factor X are almost identical with those of serum factors VII and IX. However its resistance to storage is lower. It disappears from normal serum in a few hours or a few days at room temperature. It is denatured at 50°C. Its affinity to BaSO₄ is more pronounced than that of Factor VII and IX (see method of purification). By chromatography it is irreversibly adsorbed. It is also adsorbed by BaCO₃ and Ca₃(PO₄)₂. In diluted solution (serum 1/10 with veronal acetate buffer pH 7.35) it is moderately activated at room temperature within 20 to 30 min.

Factor X compared to other recently described clotting factors. Factor X is not identical with PTA(10), the latter being only slightly adsorbed on barium sulfate. Deficiency of PTA produces an abnormal thromboplastin generation only if both BaSO₄ plasma and serum of the patient are used in the test. PTA is therefore neither adsorbed on BaSO₄ nor consumed during coagulation.

Two new clotting factors described by Spaet(11) and Ratnoff(12) are not identical with factor X because they are not adsorbed on barium sulfate.

Summary. 1. A new clotting factor, factor X, participating in blood thromboplastin formation is demonstrated by matching experiments with various pathological sera. The possible influence of thrombin and factor VII is eliminated. The objection that factor X deficiency may be nothing else than a slight deficiency of factor IX is discussed. This hypothesis is in contradiction with our results. 2. Factor X is present in normal serum and in hemophilia B-Serum, absent in hepatitis-serum and in serum of Marcoumar-(Dicoumarol derivative) treated patients. The affinity of factor X to barium sulfate is greater than that of Factor VII and IX. This property is utilised for the purification of Factor X. 3. Factor X is not identical with PTA and the new factors of Spaet *et al.* and of Ratnoff *et al.*

1. Biggs, R., Douglas, A. S., and Macfarlane, R. G., *Physiol.*, 1953, v119, 89.
2. Duckert, F., Flückiger, P., Isenschmid, H., Matter, M., Vogel-Meng, J., and Koller, F., *Acta haemat.*, 1954, v12, 197.
3. Koller, F., *Arch. exp. Path. u. Pharmacol.*, 1954, v222, 89.
4. Flückiger, P., Duckert, F., and Koller, F., *Schweiz. Med. Wschr.*, 1954, v84, 1127.
5. Duckert, F., Flückiger, P., and Koller, F., *Rev. Hematol.*, 1954, v9, 489.
6. Flückiger, P., Hässig, A., and Koller, F., *Acta haematol.*, 1954, v12, 339.
7. Koller, F., Loeliger, A., and Duckert, F., *ibid.*, 1951, v6, 1.
8. Bell, W. N., and Alton, H. G., *Nature*, 1954, v174, 880.
9. Isenschmid, H., *Acta haemat.*, 1955, v13, 177.
10. Rosenthal, R. L., *Fed. Proc.*, 1954, v13, 284.
11. Spaet, T. H., Aggeler, P. M., and Kinsell, B. G., *J. Clin. Invest.*, 1954, v33, 1095.
12. Ratnoff, O. D., and Copley, J. E., *ibid.*, 1955, v34, 602.
13. Duckert, F., *Schweiz. Med. Wschr.*, in press.

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Response of Visual Area to Callosal Impulses in the Cat.* (21928)

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There are still uncertainties concerning the colossal connections of the visual area. The anatomical(1-3) and the oscillographic data are equivocal. Reactions of the area to a synchronized volley of callosal impulses have been found in the cat by Curtis(3) and also (from his experimental map) by Garol(4). But Curtis(3), McCulloch and Garol(5), Bailey, Bonin and McCulloch(6) were unable to discover any commissural connections from area 17 in the cat, the monkey and in the chimpanzee.

In the unanesthetized cat ("encéphale isolé" préparation) callosal responses of the visual area, which can be recorded with great regularity, have revealed special features

which seem of interest.

Methods. All observations have been done on adult cats after an initial ether narcosis during which the spinal cord had been cut at the C₁ level, the optic nerve prepared on one side and the cortex exposed on both sides. The callosal volley was produced by a thyatron shock of 0.5 millisecond duration applied to a point of the contralateral gyrus lateralis symmetrical to the point whose potentials were recorded. The responses of various other areas were displayed simultaneously on the second beam of the cathode ray oscilloscope. The differential amplifier systems had an overall time-constant of 0.1 sec. The cortical stimulation was bipolar, the recording of the potentials monopolar. The response of the visual area I to shocks on the contra-

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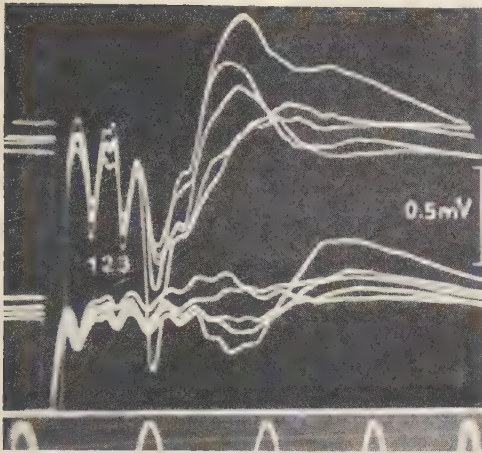


FIG. 1. A: Responses of area I (upper traces) and of suprasylvian gyrus to a shock on contralateral optic nerve (negativity upwards); 5 successive sweeps taken at 10 second intervals show lability of the slow phases of the response, contrasting with the stability of its spike-like components; time marks: 10 msec.

lateral optic nerve or to brief flashes of light were recorded for comparison.

Results. It seems opportune to recall first the characteristic pattern of the reaction of the visual area I to a volley of geniculocortical impulses. As described by Bartley and Bishop(7), the distinctive feature of the response is its beginning by a succession of surface-positive spike-like deflections, labeled 1, 2 and 3 in Fig. 1 (see also Figs. 2A, 3B), preceding the slower diphasic oscillation (positive-negative) which constitutes the reaction of all isocortical areas to a volley of afferent impulses (see Fig. 2B). Our own analysis has led us to endorse the conclusion of Chang and Kaada(8), but not confirmed by Bishop(9), that these initial spike-like deflections all represent radiation potentials assignable to the activities of different systems of geniculocortical neurones. As such these components of the response are much less labile than the following slower positive-negative complex which expresses the post-synaptic reaction of cortical neurones (Fig. 1, upper traces). This typical pattern is observed in all divisions of visual area I, with an eventual variation of amplitude of its components (Fig. 2A). Paraviscual areas (Fig. 1, lower traces) show small dips in phase with the spikes of the visual area and probably repre-

senting the field effects of radiation potentials.

The recording of the response of the same visual area to a callosal volley revealed an analogous pattern to the sensory reaction just described, and quite unlike the callosal response of other cortical regions (Fig. 2C) for the acoustic area. Like the sensory response it is characterized by an initial succession of spike-like deflections preceding the slower potential and encroaching upon it. Callosal response from 3 different experiments is reproduced in Fig. 3B, C (upper trace) and D. The analogy is particularly striking between the lower trace of Fig. 2A and the upper trace of Fig. 3C because of the small amplitude of the slow components of the responses in both cases.

Except for small field effects (Fig. 3C, lower trace), the typical response is limited to the visual area. It is completely abol-

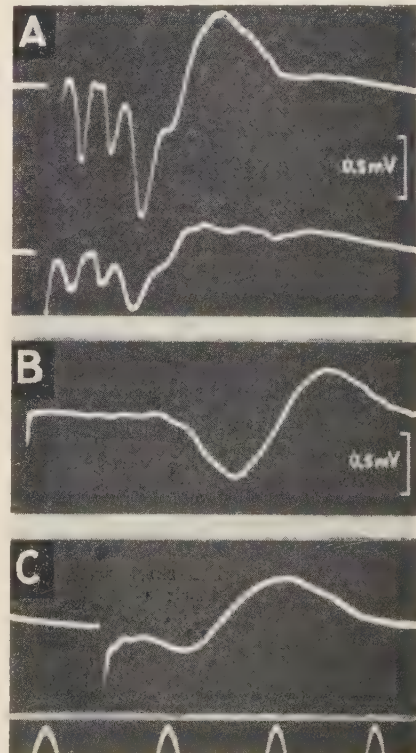


FIG. 2. A: Responses of middle portion of visual area (upper trace) and of posterior portion of visual area I, response to a click; B: auditory area I, response to a shock on symmetrical point of opposite hemisphere; time: 10 msec.

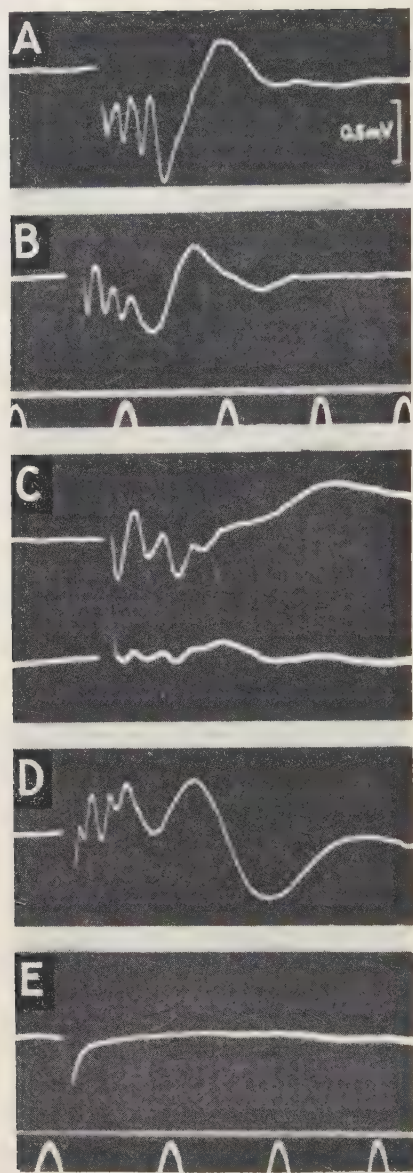


FIG. 3. A: Callosal response of visual area I; B: same cat, response of same point of visual area to optic nerve shock; C: another cat, callosal response of visual area I (upper trace) and of suprasylvian gyrus. D and E, another cat; D: callosal response of visual area I; E: complete disappearance of response after section of corpus callosum.

ished by the section of the corpus callosum (Fig. E) and it can exert on the sensory response (to an optic-nerve-shock or to a flash) following it the same facilitatory effect which has been described for the acoustic

area(10,11). The precession of a callosal volley may treble the voltage of the sensory response. As for the acoustic area, the facilitatory modification is not related to any slow wave following the callosal volley and is a long lasting phenomenon, with a regular curve of decay sometimes not ended after 150 msec.

The experimental analysis which suggests that the spike-like deflections of the sensory response are fiber potentials leads to the same conclusion for the spikes of the callosal response. Like the sensory spikes they are unaffected by a local strychnization or by a narcotic depression, which strongly affect the later slower components of the response. They are picked up from the underlying white matter after excision of the visual cortex. When the stimulating shock is applied directly on the relevant callosal fibers on the midline, the spacing of the spikes is reduced, a logical consequence of the shortening of the conduction distance for impulses which are supposed dispersed on account of their different velocities(12).

Conclusions. This being so, the formal analogy between the sensory and the callosal response of the visual area seems to indicate that the system of retino-geniculo-cortical fibers (and neurones) which is responsible for the characteristic pattern of the sensory response is prolonged beyond the primary visual cortex in the commissural fiber-tract connecting the left and right homologous areas. As a physiological interhemispheric transfer between homologous receiving areas (visual, auditory and somatosensory) has been demonstrated by psycho-physiological (13) and electro-physiological(14) experiments, the *raison d'être* of the callosal prolongation of this system seems to be looked for in the necessity of conserving the spatio-temporal pattern, and correlatively the information, of the sensory message during its interhemispheric transfer.

Summary. The reaction of the visual area in the unanesthetized cat to a synchronized volley of callosal impulses is markedly similar to the response of the same area produced by a shock on the optic nerve. Like this response, it is characterized by an initial succession of spike-like deflections preceding the slower

cortical potentials. Various controls (confirming Chang and Kaada's conclusion for the sensory response) indicate that these spike-like potentials are in both cases afferent fiber potentials, and thus represent presynaptic events for the cortical neurones. The significance of the formal analogy between the sensory and the callosal response of visual area is discussed. A callosal volley can exert on a subsequent sensory response of the visual area the same facilitatory effect which has been described by the author for auditory-callosal and -sensory impulses.

1. Bailey, F., and Bonin, G. von, *The Isocortex of Man*, Univ. of Illinois Press, 1951, Chp. VII, 234.
2. Nauta, W. J. H., and Backer, V. M. J., *Comp. Neurol.*, 1955, v100, 257.
3. Curtis, H. D., *J. Neurophysiol.*, 1940, v3, 407.
4. Garol, H. W., *J. Neuropathol. Exp. Neurol.*,

1942, v1, 422.

5. McCulloch, W. S., and Garol, H. W., *J. Neurophysiol.*, 1941, v4, 555.

6. Bailey, P., Garol, H. W., and McCulloch, W. S., *ibid.*, 1941, v4, 564.

7. Bartley, S. H., and Bishop, G. H., *Am. J. Physiol.*, 1933, v103, 159.

8. Chang, H. T., and Kaada, B., *J. Neurophysiol.*, 1950, v13, 305.

9. Bishop, G. H., and Clare, M., *ibid.*, 1951, v14, 497.

10. Bremer, F., *Rev. Neurol.*, 1952, v87, 162.

11. ———, *Arch. Int. Physiol.*, 1953, v51, 110.

12. Chang, H. T., *Research Public. Assn. f. Research in Nerv. a Ment. Dis.*, 1952, v30, 430.

13. Myers, R. E., and Sperry, R. W., *Anat. Rec.*, 1953, v115, 351.

14. Bremer, F., and Terzuolo, C., *J. de Physiol.*, 1955, v47, 105.

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Influence of Pyridoxine on Transaminase Activity of Human Placenta, Maternal and Fetal Blood.* (21929)

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The response of pregnant women to tryptophane(1,2) and pyridoxal loading tests(3) are characteristic of subjects with vit. B₆ deficiency(4,5). The abnormal excretion of xanthurenic acid after tryptophane loading can be abolished by daily administration of 10 mg of pyridoxine hydrochloride(6). This would imply that the altered tryptophane metabolism in normal pregnancy is the result of a deficiency in vit. B₆. Nevertheless, Vilter *et al.*(7) were "unable to demonstrate any other evidence of vit. B₆ deficiency by blood, urine, or tissue levels of this vitamin, or by measuring output of pyridoxic acid in urine." It would seem desirable, therefore, to search for some other evidence of vit. B₆ deficiency to justify large supplements of pyridoxine to diets of pregnant women. There is consider-

able evidence that pyridoxine derivatives, pyridoxal phosphate and pyridoxamine phosphate, serve as coenzymes of transamination reactions(8). Some tissues of pyridoxine deficient animals have a reduced transaminase activity.

Therefore, we investigated the effect of pyridoxine supplements on transaminase activity of certain fetal and maternal tissues. Blood levels were studied, inasmuch as Marsh, Greenberg and Rinehart(9) have demonstrated a correlation between pyridoxine intake and glutamic-aspartic transaminase activity of hemolyzed blood in monkeys and man. The placenta was also studied because it is the only other available fetal tissue.

Methods. Subjects were divided as follows: Group I: Ten non-pregnant healthy women who did not receive a pyridoxine supplement. Group II: Eighteen pregnant women without pyridoxine supplement.

* Supported by grants from the National Heart Institute of the National Institutes of Health, Public Health Service; and from the Christine Breon Fund.

Group III. Eight pregnant women who received 10 mg pyridoxine hydrochloride per day during the last 4-6 weeks of pregnancy.[†] Transaminase activity was measured on bloods of Groups I to III and on placentas of Groups II and III. Cord bloods as well as maternal bloods were collected in 10 subjects of Group II. Blood samples from pregnant women were collected at time of delivery, using potassium oxalate as the anticoagulant. After aliquots were removed for cell counts and hematocrit levels, the remainder was repeatedly frozen and thawed in an equal volume of distilled water. Hemolyzed blood was centrifuged lightly for one minute and the clear red supernate used for transaminase determinations. Freshly delivered placentas were drained of blood, placed in plastic bags and stored in frozen state. Breis were prepared by dissection and homogenization for 20 seconds in a cold Waring Blendor. Undiluted breis were routinely freed of connective tissue and frozen before further extraction. Frozen breis retained their original activity for several months despite repeated freezing and thawing. At the time of determination, 0.5 g of freshly thawed brei were ground in a cold mortar and transferred to a graduated centrifuge tube with small portions of ice cold 0.1 M phosphate buffer, pH 7.4. The final volume was adjusted to 10 ml. The homogenate was centrifuged lightly for one minute and 0.2 ml of supernate used in each tube for transaminase determinations. Glutamic-aspartic transaminase activity was determined by a modification of the method of Tonhazy, White and Umbreit(10). The reagents were the same as those of the original method except that trichloroacetic acid was omitted because of high blank values in its presence. Instead, enzyme reaction was stopped with aniline citrate to which had been added hydrochloric acid equivalent to the trichloroacetic acid omitted. Blanks and standards contained the same amount of homogenate and substrates as did the reaction mixtures. The substrates (and pyruvate in the

TABLE I. Transaminase Levels in Blood.

Group	No. in group	Transaminase activity (μ l oxalacetate)
I. Non-pregnant women	10	734 \pm 129*
II. Pregnant women	18	748 \pm 173
III. <i>Idem</i> + 10 mg pyridoxine HCl daily	6	984 \pm 129
IV. Cord bloods [†]	10	1467 \pm 194

* Means and stand. dev. of observations.

[†] From babies of 10 of the women of Group II.

standards) were added *after* the aniline citrate reagent in the blanks and standards. Inordinately high blanks were obtained if alpha-ketoglutaric acid was omitted. It was necessary to use the means of triplicate determinations for blanks, standards and reaction mixtures to obtain consistent results. Some of the difficulties encountered with this method have been discussed by Marsh, Greenberg and Rinehart(9). Preliminary investigation indicated that Q_T^{10} , as defined by Tonhazy, *et al.* (10), best expressed enzyme activity for comparison between placentas. The activity of blood was expressed as the number of μ l of oxalacetate formed per hour per ml of blood, calculated from the amount of pyruvate obtained after incubation of 0.1 ml of blood for 10 minutes.[‡]

Results. Levels of transaminase in blood samples are shown in Table I. Pregnancy appeared to have no effect. Addition of 10 mg of pyridoxine hydrochloride to the daily diet of pregnant women for several weeks resulted in a significant increase in transaminase activity ($P < 0.01$). In contrast to effect on blood activity, supplements of pyridoxine had no effect on the transaminase activities of the placentas. The mean Q_T^{10} values with standard deviations were 37.9 ± 6.2 and 35.3 ± 6.0 for the supplemented and unsupplemented groups respectively. Cord bloods all contained high concentrations of transaminase activity. There was no correlation between blood levels of mothers and those of their infants. Although transaminase activity does exist in plasma(11), the method used is not sensitive enough for its measurement. Therefore, the

[†] We are indebted to the Stuart Co. for a generous supply of their Prenatal Capsules, some of which were specially prepared free of pyridoxine.

[‡] Units, expressed as μ g of sodium pyruvate/10 min./ml of blood(9), multiplied by the factor 1.22 correspond to the units used here.

TABLE II. Red and White Blood Cell Counts. Ten patients in each group.

Group	R. B. C. (millions)	W. B. C. (thousands)
I. Non-pregnant women	$4.44 \pm .8^*$	8.2 ± 1.8
II. Pregnant women (during delivery)	4.74 ± 1.1	16.0 ± 3.4
IV. Cord bloods	$4.76 \pm .4$	16.4 ± 3.7

* Means and stand. dev.

variations noted must have occurred in activities of individual cells, or in the number of cells, or both. The red cell counts of cord bloods did not differ significantly from those of any other group, nor did the white cell counts of cord bloods differ from those of mothers, although both were twice those of the non-pregnant controls (Table II). It would, therefore, appear that the activity of fetal blood cells was exceptionally high, when compared to maternal or non-pregnant subjects.

Transaminase activity of blood could be influenced very rapidly by raising the level of pyridoxine ingestion. This was demonstrated in the case of a patient hospitalized for mild toxemia (Table III). After transaminase activity had been determined on a control blood sample, 150 mg of pyridoxine hydrochloride per day were given by mouth. On the third day of supplementation, the transaminase activity showed an increase of 81%, and 32 hours later, an increase of 120%. Transaminase activity of cord blood fell within the range of those from unsupplemented mothers.

Discussion. The average American daily diet contains from 1.4 to 1.7 mg of pyridoxine (12). On the basis of observations of Marsh, Greenberg and Rinehart(9), it would be expected that a true deficiency of vit. B₆ should result in blood transaminase levels signifi-

cantly lower than those found in healthy non-pregnant subjects. No significant difference was found as the result of normal pregnancy. Pregnant women responded to B₆ supplements with increased levels of blood transaminase, but this is equally true in non-pregnant subjects(9).

Placental transaminase activity was not influenced by the pyridoxine supplements. This could mean that glutamic-aspartic transaminase activity of the human placenta, like that of the adult rat liver(13), is relatively insensitive to profound changes in vit. B₆ concentration, or more likely, that it has accumulated from maternal sources an optimal supply of pyridoxine. Transaminase activity of term placenta, expressed as units/mg of solids, is considerably higher than fetal cord blood, which itself is much higher than maternal blood.

With respect to fetal blood, the data suggest that the infant's transaminase system is well supplied with pyridoxal or pyridoxamine cofactors, perhaps at the mother's expense. If this is a "desirable situation" for the fetus, it might be reasoned that it should be desirable for the adult. The pregnant woman may be able to maintain a "normal" blood transaminase level, by non-pregnant adult standards, and still have insufficient reserve on which to draw under such conditions of stress as tryptophane loading. If one assumes, however, that increasing the transaminase level of maternal blood by pyridoxine supplements is desirable, the same reasoning would hold true for the non-pregnant adult population.

Summary. 1. The glutamic-aspartic transaminase activity of whole blood from normal pregnant subjects is essentially the same as in non-pregnant subjects. Activity is significantly increased by supplementing the diet with 10 mg of pyridoxine daily. Transaminase activity of fetal blood is twice as great as in maternal blood in mothers not receiving additional vit. B₆. The transaminase activity of placental tissue is not altered by prior administration of pyridoxine for several weeks. 2. The results suggest that fetal tissues contain optimal quantities of B₆, whereas adults, both pregnant and non-pregnant, contain sub-optimal concentrations for peak enzymatic

TABLE III. Effect of Pyridoxine Supplements on Blood Transaminase in the Same Individual.

Time of sample	Transaminase (μ l oxalacetate)	R. B. C. (mill.)	W. B. C. (thous.)
8/10/54 (control level)	390	4.54	9.4
8/13/54	661	4.46	9.4
8/14 *	855	4.88	18.4
Cord blood	1660	5.10	20.8

* Immediately after delivery of baby.

activity. The method employed is not sufficiently sensitive to demonstrate a reduced "reserve" of B₆ in normal gestation, but the data are compatible with the view that pyridoxine supplementation is desirable in human pregnancy.

1. Sprince, H., Lowy, R. S., and Folsome, C. E., *Am. J. Obst. and Gynec.*, 1951, v62, 84.
2. Wachstein, M., and Gudaitis, A., *J. Lab. and Clin. Med.*, 1952, v40, 1.
3. ———, *Am. J. Obst. and Gynec.*, 1953, v66, 1207.
4. Greenberg, L. D., Bohr, D. F., McGrath, H., and Rinehart, J. F., *Arch. Biochem.*, 1949, v21, 237.
5. Rabinowitz, J. C., and Snell, E. E., *PROC. SOC. EXP. BIOL. AND MED.*, 1949, v70, 235.
6. Wachstein, M., and Gudaitis, A., *J. Lab. and Clin. Med.*, 1953, v42, 98.
7. Vilter, R. W., Biehl, J. P., Mueller, J. F., and Friedman, B. I., *Fed. Proc.*, 1954, v13, 776.
8. Snell, E. S., *Physiol. Rev.*, 1953, v33, 509.
9. Marsh, M. E., Greenberg, L. D., and Rinehart, J. F., *J. Nutrition*, 1955, v56, 115.
10. Tonhazy, N. E., White, N. G., and Umbreit, W. W., *Arch. Biochem.*, 1950, v28, 36.
11. Karmen, A., Wroblewski, F., and LaDue, J. S., *J. Clin. Invest.*, 1955, v34, 126.
12. Booher, L. E., and Behan, I. T., *J. Nutrition*, 1949, v39, 495.
13. Beaton, J. R., Beare, J. L., Beaton, G. H., Caldwell, E. F., Ozawa, G., and McHenry, E. W., *J. Biol. Chem.*, 1954, v207, 385.

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Effect of 2,4-Dinitrophenol on Sea-Urchin Sperm. (21930)

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2,4-Dinitrophenol (DNP) has a similar effect on many metabolic systems(1), stimulating respiration and inhibiting energy-requiring processes in low concentrations and inhibiting respiration as well as endergonic processes in high concentrations. Lardy and Phillips(2) showed that DNP stimulates respiration of bull sperm and reduces sperm motility. Since metabolism of sea-urchin sperm differs in several respects from that of mammalian sperm(3,4), it was of interest to examine the effect of DNP on sea-urchin sperm. Dinitrophenol stimulates respiratory rate and causes a reversible block to cleavage in sea-urchin eggs(5,6), but no investigations have been reported concerning the effect of DNP on sea-urchin sperm. Sperm of sea urchins and other animals exhibit "Dilution Effect"(4), characterized by rapid decrease in length of motile life and fertilizing capacity of sperm, and by temporary rise in respiratory rate, upon dilution of the suspension, the rate then falling off toward zero as sperm lose their motility. Metal-chelating agents, such as

glycine and ethylenediamine tetracetic acid (Versene), can largely abolish the symptoms of the "Dilution Effect"(7-9), *i.e.* they extend the life span of sea-urchin sperm and suppress the temporary rise in respiratory rate, maintaining respiration at a low, relatively steady level.

It was, therefore, of interest to examine the effect of DNP on sea-urchin sperm in the presence of a metal-chelating agent such as Versene.

Materials and methods. Sperm from the sea urchin *Strongylocentrotus purpuratus* were used. Oxygen consumption was measured with the Warburg respirometer. Dry semen was diluted to a concentration of 4×10^8 to 6×10^8 cells per ml with sea water or with $10^{-3}M$ Versene[†] in sea water, and 3 ml of these suspensions were placed in the main compartments of 25 ml Warburg flasks; the gas space in the flasks contained air; CO₂ was absorbed with alkali. Experiments were done at 17.8°C. Dinitrophenol (0.3 ml) was added to sperm suspensions from magnetically control-

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[†]I am indebted to Versenes Inc., Framingham, Mass., for samples.

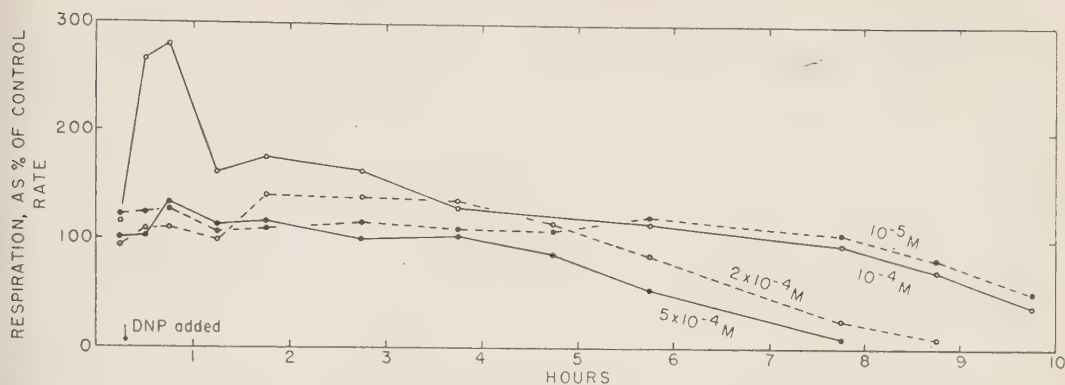


FIG. 1. Effect of DNP on *S. purpuratus* sperm suspended in 10⁻³M Versene. Curves were determined in separate experiments; respiratory rates were averaged for duplicate flasks.

led cups to give the final concentrations mentioned below. The pH of Versene and DNP solutions was adjusted to that of sea water (pH 8.00 to 8.05), and the osmotic pressures were approximately that of sea water. Triplicate test and control flasks were run in each experiment. Two flasks of each set were used for measuring oxygen consumption, and sperm motility observed periodically by removing samples from the third flask.

Results. At 10⁻⁴M, 10⁻⁵M, and 10⁻⁶M, DNP had no appreciable effect on respiration or motility of sperm suspended in sea water. At concentrations between 2 x 10⁻⁴M and 5 x 10⁻⁴M, DNP inhibited respiration and motility, the degree of inhibition increasing as the concentration of DNP was increased. Inhibition of motility could be reversed by washing and resuspending the treated sperm in fresh sea water.

The response of some systems (including bull sperm) (10) to DNP depends on the initial respiratory rate of the system (1), *i.e.*, the lower the respiratory rate, the more it can be stimulated by DNP. Sea-urchin sperm have a relatively high respiratory rate when suspended in sea water because of the "Dilution Effect." Thus, it seemed possible that if the respiratory symptom of the "Dilution Effect" were suppressed, DNP might then stimulate sperm respiration. Respiration was controlled by suspending the sperm in 10⁻³M Versene (9), the sperm in this diluent having an initial respiratory rate from 25% to 65% of the initial rate of sperm in sea water.

Sperm in Versene were treated with 10⁻⁵M,

10⁻⁴M, 2 x 10⁻⁴M, and 5 x 10⁻⁴M DNP. At 10⁻⁴M, DNP initially stimulated respiration to 280% of the control rate (Fig. 1), the respiratory rate of the treated sperm falling below the control rate within 8 hours. The other concentrations of DNP gave less stimulation, and respiration fell below the control rate more quickly as concentration of DNP was increased. Except at 5 x 10⁻⁴M, DNP did not detectably inhibit sperm motility until after the respiratory rate of treated sperm fell below that of controls. At 5 x 10⁻⁴M, DNP inhibited the motility of Versene-treated sperm by about 40% (visual estimation) within 20 minutes, and the life span of treated sperm was shorter than that of control sperm (Fig. 2).

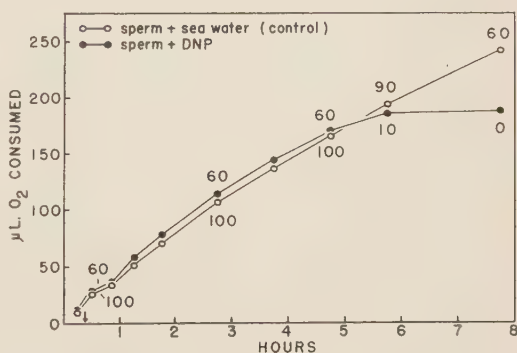


FIG. 2. Inhibition of motility and dissociation of respiration from motility by 5 x 10⁻⁴M DNP, sperm in Versene (10⁻³M). Points represent averages for duplicate flasks. Numerals represent motility ratings (visual estimation), initial motility rating = 100. Arrow indicates when DNP was added to sperm. (After curves cross, motility ratings are still placed next to curve to which they refer.)

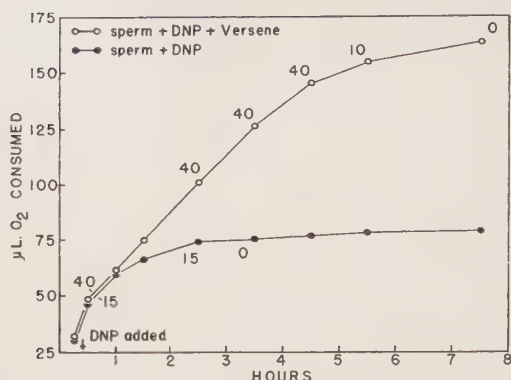


FIG. 3. Protective effect of Versene ($10^{-3}M$) on *S. purpuratus* sperm in $5 \times 10^{-4}M$ DNP. Points represent averages for duplicate flasks. Numerals represent motility ratings (visual estimation), initial motility rating = 95.

Versene, as compared to sea water, had a protective effect on sperm treated with $5 \times 10^{-4}M$ DNP (Fig. 3). When DNP was added to sperm in sea water motility was inhibited within 15 minutes, and the sperm rendered completely immotile within 3 hours. Versene, added simultaneously with DNP, reduced the initial inhibition of motility and prolonged the life span and respiration of DNP-treated sperm for 3 to 5 hours.

Unbuffered sea water was used in these experiments, because buffers that might have been used, such as glycylglycine(11), would be likely to affect respiration by virtue of chelating or other specific effects. As a result, the pH of the sperm suspensions in the flasks increased with time (as sperm respiration declines and less respiratory CO_2 is produced, the KOH in the alkali cup begins to extract CO_2 from the sea water diluent). Since DNP penetrates cells more easily as an undissociated molecule than as an ion(12), pH changes alter the effective concentration of the inhibitor. In these experiments, however, the pH of the sperm suspensions in the Warburg flasks did not change appreciably for 1 to 3 hours, indicating that the differential effect of DNP in the presence and absence of Versene was not due to differences in pH of the suspending media.

Discussion. Dinitrophenol at low concentrations can inhibit energy-requiring processes by blocking oxidative phosphorylation and thereby reducing the amount of adenosine-

triphosphate available to the cell(13). Since respiration is stimulated as this inhibition occurs, DNP, in effect, "uncouples" respiration from endergonic metabolism. Dinitrophenol apparently effects some "uncoupling" of respiration and motility with *S. purpuratus* sperm. Thus, at $5 \times 10^{-4}M$ DNP inhibits motility while slightly stimulating respiration (Fig. 2). At lower concentrations, however, DNP does not inhibit motility as it stimulates respiration, but "uncoupling" occurs in the sense that the increase in respiratory rate is not accompanied by an increase in sperm activity.

In addition to inhibiting oxidative phosphorylation, DNP also stimulates the adenosine triphosphatase (ATPase) activity of some tissues(14), the two effects sometimes being interrelated(13). It is interesting, then, that Versene and DNP have the same qualitative effect on the activity of rat-brain ATPase(15) as they have on the respiration of sea-urchin sperm. Thus, DNP stimulates the ATPase activity of rat-brain homogenates, but only after the initially high activity of the preparations has been reduced by treatment with ethylenediamine tetracetate.

The effect of DNP on respiration and motility of sea-urchin sperm may involve both an inhibition of oxidative phosphorylation and a stimulation of sperm ATPases.

It is apparent that DNP and dilution have similar effects on *S. purpuratus* sperm. Both shorten the life span and stimulate respiration of sperm, DNP not having a stimulatory effect until that of dilution has been abolished by Versene. Also, both seemingly cause some dissociation of respiration and motility; for dilution this is suggested by the fact that Versene reduces the high respiratory rate of diluted sperm without reducing sperm motility.

As the concentration of DNP is increased, DNP and the agent responsible for the "Dilution Effect" seem to have an accumulative inhibitory effect on sperm respiration and motility.

These similarities raise the possibility that the effects of dilution and DNP occur through similar mechanisms. Thus the "Dilution Effect" may involve an inhibition of oxidative phosphorylation or an increase in the activity

of a sperm ATPase, perhaps one not associated with motility.

Summary. Low concentrations of DNP did not stimulate respiration or affect motility of sea-urchin sperm suspended in sea water; high concentrations of DNP inhibited sperm respiration and motility, the inhibition of motility being reversible. Dinitrophenol stimulated the respiratory rate of sperm in the presence of Versene and caused respiration to be partially dissociated from motility. Versene, as compared with sea water, prolonged the motile life and respiration of sperm treated with high concentrations of DNP. Dinitrophenol and dilution have similar effects on sea-urchin sperm, raising the possibility that both act through similar mechanisms.

1. Simon, E. W., *Biol. Rev.*, 1953, v28, 453.
2. Lardy, H. A., and Phillips, P. H., *J. Biol. Chem.*, 1943, v149, 177.
3. Mann, T., *The Biochemistry of Semen*, Methuen and Co., London, 1954.

4. Rothschild, Lord, *Biol. Rev.*, 1951, v26, 1.
5. Clowes, G. H. A., and Krahle, M. E., *J. Gen. Physiol.*, 1936, v20, 145.
6. Tyler, A., and Horowitz, N. H., *Biol. Bull.*, 1938, v75, 209.
7. Tyler, A., and Rothschild, Lord, *Proc. Soc. Exp. Biol. and Med.*, 1951, v76, 52.
8. Tyler, A., *Biol. Bull.*, 1953, v104, 224.
9. Rothschild, Lord, and Tyler, A., *J. Exp. Biol.*, 1954, v31, 252.
10. Melrose, D. R., and Turner, C., *Biochem. J.*, 1953, v53, 296.
11. Tyler, A., and Horowitz, N. H., *Science*, 1932, v86, 85.
12. Tyler, A., *Proc. Nat. Acad. Sci.*, 1937, v23, 369.
13. Hunter, F. E., Chap. 5 in *Symposium on Phosphorus Metabolism*, v1, Johns Hopkins Press, Baltimore, 1951.
14. Lardy, H. A., and Wellman, H., *J. Biol. Chem.*, 1953, v201, 357.
15. Maxwell, R. E., and Nickel, V. S., *Proc. Soc. Exp. Biol. and Med.*, 1954, v86, 846.

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Effect of Age on Polysaccharide Composition of Cartilage. (21931)

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Human cartilage has been subjected to chemical analysis by Hass(1) who studied the effect of aging on its composition. His methods for studies of polysaccharide components consisted of the determination of reducing substances and of sulfate in washed cartilage after hydrolysis with 4.2 N HCl. All of the sulfate and one-half of the reducing substances were assumed to be derived from chondroitin-sulfuric acid under these circumstances. As methods for hexosamine, hexose, and uronic acids are now available and have been used in this laboratory for studies of tissue, it is possible to make more specific studies of the polysaccharide moieties of cartilage. The following study was accordingly initiated.

Methods. Costal cartilage samples were

obtained at autopsy from patients who had died from a number of different pathological conditions. The cartilage was freed from extraneous tissue, cut in fine pieces with a surgical knife and dried in a vacuum oven at 60°C. The dried cartilage was ground to pass through a 60 mesh screen.

Uronic acid was determined by a method which was modified from the carbazole method of Dische(2). Ten mg samples of cartilage were weighed into glass stoppered test tubes (16 x 150 mm). Ten ml of 85.7% by volume of sulfuric acid (concentrated H₂SO₄; Sp. Gr. 1.84, diluted 6:1 with distilled water) were added and the tubes placed in a boiling water bath for 15 minutes. After cooling 2 ml aliquots were pipetted into each of 3 glass stoppered test tubes. Twelve ml of 85.7% sul-

TABLE I. Uronic Acid and Hexosamine Contents* of Human Costal Cartilage.

Age group	No.	Uronic acid	Chondroitin sulfate†	Hexosamine	
				Total	Excess‡
Fetal	5	7.8 (7.4-8.0)	19.1	8.2 (7.9-8.5)	1.0 (.6-1.1)
Newborn	2	7.5 (7.3-7.7)	18.4	8.7 (8.5-8.9)	1.3 (1.2-1.4)
4 mo	1	7.0	17.2	7.1	1.6
6-11 yr	2	3.6 (3.6-3.7)	9.0	6.1 (6.0-6.2)	2.7 (2.6-2.8)
25-40 "	5	3.3 (2.9-3.9)	8.0	7.3 (6.9-7.7)	4.3 (3.4-4.6)
41-55 "	5	2.4 (2.0-3.2)	5.8	6.8 (5.4-7.7)	4.6 (3.9-5.1)
56-70 "	10	1.6 (1.3-1.9)	4.0	6.1 (4.9-6.6)	4.6 (3.6-5.0)
71-88 "	4	1.3 (1.1-1.6)	3.2	5.4 (4.7-6.0)	4.3 (3.6-4.6)

* All results are expressed as % of dry material.

† Calculated from uronic acid data using factor 2.46.

‡ Hexosamine in excess of that calculated to be in the chondroitin sulfate.

furic acid were added to each tube. The tubes were cooled 10 minutes in an ice bath. 0.4 ml of carbazole reagent (0.1 g/100 ml absolute ethanol) was added to 2 of the tubes; 0.4 ml of absolute ethanol was added to the third tube which served as an experimental blank. The tubes were mixed by inversion and placed in a 37°C incubator for 1 hour and then in a 30°C water bath for 1 hour. Samples were then read in a Coleman Spectrophotometer at 530 mμ against the respective blank. A standard solution of glucuronic lactone* equivalent to 100 μg of glucuronic acid in 2 ml of water was cooled 10 minutes in an ice bath and then diluted with 12 ml of concentrated H₂SO₄. It was hydrolysed for 20 minutes in the boiling water bath. After cooling for 10 minutes in the ice bath, the carbazole reagent was added and the tubes were carried through the procedure as described above. This standard was read against a blank containing glucuronic lactone, sulfuric acid and absolute alcohol, subjected to the same procedure. Hexosamine was determined by a modified Elson-Morgan method as previously described by Shetlar, *et al.*(3).

Results. Results of analytical determinations are summarized in Table I. It can readily be seen that uronic acid is quite high in young cartilage, but decreases drastically in cartilage of children and mature individuals and decreases even more in old age. Hexosamine content also decreases with age, but the decrease is much less striking than it is for uronic acid. If one assumes that all the uronic acid of cartilage is contained in chon-

droitin sulfuric acid, the latter component may be calculated from the uronic acid content. The excess hexosamine may logically be assumed to be contained in neutral polysaccharides with hexosamine and hexose as carbohydrate constituents. These calculated data indicate that although the acid polysaccharide of cartilage decreases with age, neutral polysaccharide increases in cartilage until maturity. After maturity no appreciable change of neutral polysaccharide occurs.

Discussion. The chondroitin sulfate values reported in Table I are in general somewhat lower than those reported by Hass(1). These differences may be due to the different methods used to arrive at the values found for chondroitin sulfate. Hass assumed that one-half the reducing substance and all of the sulfate of cartilage was derived from the chondroitin sulfate. Obviously these are only approximations. It is noteworthy that results obtained by isolation as reported by Hass are much lower, cartilage from 35, 46, 50, and 66-year-old individuals having 11.4, 8.5, 6.9, and 5.8% of chondroitin sulfate, respectively. These results compare more favorably with the data of Table I. The other possibility is that the uronic acid determinations made as described above are too low. In the presence of some proteins the carbazole method does give results which are too low(2). However, optical densities of glucuronic acid lactone solutions carried through the analytical procedure described above were not affected by the presence of gelatin in concentrations equivalent in nitrogen content to that found in cartilage. Further studies of the carbazole method are in progress.

* General Biochemicals, Chagrin Falls, O.

In any case the data of Table I indicate chondroitin sulfate is higher in young cartilage and decreases with age. Hass reported an increase between ages 2 and 21 followed by a gradual decline. The data obtained in this study confirmed the gradual decline after 21 years, but were insufficient between the ages of 2 and 21 to confirm or fail to confirm the observation of an elevation between these ages. Hass' studies did not include cartilage samples from fetal or of newborns which have a uronic acid content much higher than that of children. When such data are considered, it appears that a high acid polysaccharide level is characteristic of young growing cartilage and may be correlated with active proliferation. A more rapid fixation of sulfate in young tissue has been reported(4,5). This fixation in part is probably a reflection of a higher chondroitin sulfate content.

Conclusions. A study was made of the polysaccharide constituents of cartilage from

individuals of various ages. Fetal cartilage was found to be very high in uronic acid. The uronic acid content was strikingly lower by the sixth year and then declined slowly with age. Hexosamine in excess of uronic acid increased with age until maturity was reached after which there was no appreciable change.

The authors are indebted to Dr. C. D. Tool, Pathologist, VA Hospital, for samples of adult cartilage and to Miss Jeanne Green, Pathology Department, University of Oklahoma School of Medicine for samples of fetal cartilage.

1. Hass, George M., *Arch. Path.*, 1943, v35, 275.
2. Dische, S., *J. Biol. Chem.*, 1947, v167, 189.
3. Shetlar, M. R., Foster, J. V., Kelly, K. H., and Everett, M. R., *PROC. SOC. EXP. BIOL. AND MED.*, 1948, v69, 507.
4. Layton, L. L., *Cancer*, 1950, v3, 725.
5. Layton, L. L., Denko, C. W., Scapa, S., and Frankel, D. R., *ibid.*, 1952, v5, 405.

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Complement-Fixing Antigens in Concentrates of Streptococcal Culture Supernates.* (21932)

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Recent research on the etiology of rheumatic fever has been directed at the only known association of this disease with any microorganism—association with hemolytic streptococcus. This relationship, which has been reviewed in terms of epidemiologic(1), serologic(2,3) and bacteriologic(4) data, suggested to several investigators that some immunologic relationship might exist between a substance derived from the hemolytic streptococcus and the tissues of the rheumatic host. Within the area of metabolic products of the streptococcal cell there has been considerable study of those antigens which have some bio-

logic or biochemical activity, and to which antibodies can be measured in neutralization tests. Such streptococcal antigens include the hemolysins, hyaluronidase, desoxyribonuclease and kinase (plasminogen activator). Other possible antigens, to which antibodies would be measurable by aggregation phenomena, have not been studied except for 2 studies of hemagglutination reactions with streptococcal supernate concentrates(5,6). The present report concerns an investigation of this potential group of streptococcal antigens.

Methods and materials. *Medium.* Streptococci were cultivated in either of 2 media, a dialysate medium and, for the most part, in the synthetic medium described by Bernheimer and Pappenheimer(7). The dialysate

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medium was prepared by dissolving dehydrated dextrose broth (Difco) at 20 times the concentration used in preparing ordinary medium and dialyzing this solution in cellophane bags against 19 volumes of distilled water in a rotating dialyzer for 18 hours at 4°C. The pH of the resulting dialysate, (6.5), was brought to 7.2 by addition of molar NaOH. After sterilization in the autoclave, one-tenth volume of sodium bicarbonate was added. This medium, or the synthetic medium, was seeded with an overnight culture of β -hemolytic streptococci, the inoculum being 4% of the volume of the medium. *Concentration of culture supernates.* The combined cultures of each day were pooled, after adjustment of the pH to approximately its original value, distributed into cellophane bags hung on a rack before an electric fan for concentration by pervaporation. After 16 hours of pervaporation at about 12°C, the contents of the bags, now concentrated approximately 10-fold, were pooled and subjected to vigorous centrifugation (30 minutes at 15,000 r. p. m.) to remove bacterial cells. This concentrate was dialyzed overnight against running tap water and then against distilled water, after which the volume was further reduced by pervaporation for 6 hours at 12°C. The pooled concentrates were then desiccated from the frozen state, and the dried material was stored at 4°C. Similar preparations could be obtained by ammonium sulfate precipitation of Seitz filtrates of the cultures but this method produced unsatisfactory yields because of loss of antigenic material by adsorption on the asbestos pad. The strains of β -hemolytic streptococci of Lancefield Group A, were obtained through the courtesy of Dr. Rebecca C. Lancefield. They were maintained in lyophilized form for use in seeding cultures as described above. The human sera obtained from presumably healthy adults in the area of Philadelphia were outdated sera supplied through the courtesy of the Philadelphia Serum Exchange.

Technic of the complement-fixation test. The complement-fixation test was the serologic test used in almost all the work described. When used for estimation of antigenic material, the solution of the latter was

prepared in various dilutions, usually in 2-fold steps of dilution, in 0.4-cc volumes. These dilutions were incubated 45 minutes at 37°C with 0.1 cc of a standard serum, diluted to its optimal concentration for this test, and 0.1 cc of guinea pig serum, diluted to contain 1.3 units of complement in this volume. After incubation each tube received 0.1 cc of 4% sheep erythrocytes (adjusted so that 1 cc of the suspension, hemolyzed and diluted to a final volume of 20 cc, yielded a reading of 135 in the Klett photoelectric colorimeter with a #54 filter), and 0.1 cc of rabbit anti-sheep-erythrocyte serum diluted to an optimum concentration for this hemolytic system. For the measurement of antibody the same system was employed, with serial dilutions of serum, and a constant amount of antigen preparation.

Results. A. Production of complement-fixing antigen(s). 1. *Observation of complement-fixing antigens in concentrates of streptococcal culture filtrates.* Concentrates of the supernates of β -hemolytic streptococcal cultures (Group A, type 4, strain H44) in both dialysate and synthetic media were tested by complement fixation in block titration against sera of rabbits, given long courses of injections of killed, and then living streptococcal cells. These rabbits had received streptococci of type 1. Typical results of such complement fixation tests are shown in Table I. It can be seen that a complement fixation reaction is demonstrable between concentrates of streptococcal culture filtrates and rabbit antistreptococcal serum. Antigenic material of streptococcal origin was designated CSA (culture supernate antigens). In exploratory work with these preparations, a small percentage of sera obtained from healthy adults contained complement-fixing antibodies to the streptococcal CSA in a range of titers from those barely measurable by the technic described here up to 32. The relatively higher-titered sera among these were used as reagents for the measurement of CSA, reported below. Table I also shows the results of a complement-fixation test of the same preparations of streptococcal CSA against such a human serum.

Uninoculated synthetic medium was concentrated as described above. The yield of

TABLE I. Complement-Fixation Tests of Streptococcal CSA Produced on Broth-Dialysate Medium and on Synthetic Medium Versus Rabbit Antiserum and Human Serum.

	CS2E (dialysate medium)					CS26 (synthetic medium)					
	Concentration, γ /cc					Concentration, γ /cc					
	250	125	63	31	16	31	16	8	4	2	0
Human serum C											
Diluted: —	0	0	0	1+	2+	0	0	0	2+	2+	2+
1:2	0	0	0	1+	3+	0	0	tr	2+	3+	3+
1:4	0	0	2+	3+	3+	0	2+	3+	3+	3+	3+
1:8	1+	3+	3+	3+	3+	3+	3+	3+	3+	3+	3+
Rabbit antiserum 232											
Diluted: 1:8	0	0	0	2+	3+	0	0	tr	2+	3+	3+
1:16	0	0	0	2+	3+	0	0	0	1+	3+	3+
1:32	0	0	1+	3+	3+	0	0	2+	3+	3+	3+
1:64	0	1+	3+	3+	3+	0	3+	3+	3+	3+	3+
—	3+	3+	3+	3+	3+	3+	3+	3+	3+	3+	3+

Degrees of hemolysis: 0, none; 1+, weak; 2+, strong; 3+, complete.

dry weight was 0.4 mg per liter, of the order of one-fortieth the yield of dry weight of CSA per liter of culture. Complement-fixation tests as described above were entirely negative, when this material was used in the same order of concentration as shown for CSA in Table I.

2. *Production of CSA by various types of streptococci of Group A.* To obtain data as to breadth of reactivity of the antigenic material and extent of its production by various types, 6-liter cultures were grown of each type, of a collection of 36 types available. The filtrate of each culture was concentrated, lyophilized and tested for its content of CSA by complement fixation against the rabbit serum mentioned above. In the available strain of each of 5 types, 6, 12, 13, 23, 37, no CSA was found within the threshold of sensitivity of our measurements. In concentrates derived from the other 31 types, CSA was found in a range of concentration from 1 complement-fixing unit per mg of lyophilized material to 16, in the case of our strain of types 25, 30, 36, and 40.

3. *Production of CSA in relation to growth of organisms.* The relationship of concentration of CSA to growth of streptococci was studied as follows: Cultures of several types were seeded into dialysate medium, and at various intervals (0, 4, 8, 12, 24 hours) samples of the well mixed cultures were withdrawn into tubes for immediate centrifugation. The sediment was washed once with

saline solution by centrifugation and resuspended in the original volume of the sample for a measurement of turbidity (Klett densitometer, #54 filter). The supernate was dialyzed overnight against running tap water in a $\frac{1}{4}$ inch cellophane tube and then concentrated by pervaporation to half the original volume. This concentrated culture filtrate was used for the measurement of CSA by complement fixation. In some cultures it was found that the greatest part of the rise in concentration of CSA occurred before the 8th hour of culture, at which time CSA concentration was 1.5 units/cc, and the turbidity of the resuspended bacterial sediment 65; 4 hours later the CSA concentration had risen to $1\frac{1}{2}$ times their value at 8 hours, and the turbidity of the bacterial sediment to 152. Thereafter there was no substantial increase in the value of either quantity. In other cultures it was found that up to 12 hours the increase in the CSA concentration was in general parallel to the increase in the turbidity of the resuspended bacterial sediment, but thereafter the latter increased without corresponding increase in CSA concentration.

B. *Characterization of CSA.* 1. *Effects of pH.* Experiments to determine the effects of high and low pH on activity and solubility of CSA were carried out on solutions of lyophilized concentrates derived from cultures in the synthetic medium. Samples of such solutions were brought to given values of pH

TABLE II. Effect of Previous Incubation with Erythrocytes on Hemolytic and Complement-Fixing Activity of a Preparation of CSA.

	Incubation mixture, 5 ml of 0.4% CSA 82, incubated with														
	A					B					C				
	.6 ml PSS (control)					.6 ml packed RBC and 10 mg NaHSO ₃					.6 ml packed RBC				
	1	2	4	8	16	Tested at dilution of					1	2	4	8	16
Complete fixation vs. rabbit antiserum No. 1685 at dilutions:															
64	0	0	0	tr	2+	0	0	0	1+	2+	0	0	0	1+	2+
128	0	0	0	tr	2+	0	0	0	1+	2+	0	0	0	1+	2+
256	1+	1+	1+	1+	3+	1+	2+	2+	2+	3+	1+	2+	2+	3+	4+
Hemolytic activity vs. 1 vol. of 0.4% RBC	4+	4+	4+	4+	1+	0	0	0	0	0	4+	4+	4+	1+	1+*

* NaHSO₃ added to this preparation for the hemolysin test.

Degrees of hemolysis: 0, none; 1+, weak; 2+, strong; 3+, almost complete; 4+, complete.

by addition of normal hydrochloric acid or ammonium hydroxide, the reagents being added dropwise to the chilled solution with constant stirring. The solutions were kept overnight at 4°C, at the desired pH, after which any precipitate which had formed was removed by centrifugation. Sediments were dissolved in a volume of buffered saline solution equal to that of the original volume, and the pH of the supernate was restored to its original value. Each fraction was then tested for CSA activity by complement fixation, in comparison with the original solution. Between pH 5 and 8.5 there was no precipitation and no loss of activity. At pH 4 a flocculent precipitate was obtained, and the activity of CSA was divided approximately equally between sediment and supernate. At pH 3.5 a more bulky precipitate was obtained, and all of the original activity could be found in the redissolved sediment, no antigen being detectable in the supernate. At pH 1 there was no activity in the supernate, and only half activity could be recovered from the redissolved sediment. At pH 9 a precipitate was obtained, but all serologic activity of the original material could be found in the supernate. At pH 10 a larger, inactive, precipitate was obtained and some loss of activity of the supernate was found. The extent of precipitation of inactive material at pH 9 was not sufficient to increase measurably the activity per mg of the preparation under present conditions of testing.

2. *Precipitation by ammonium sulfate.* A 4% solution of CSA was gradually brought to 50% of saturation with ammonium sulfate by equilibrating the solution with one volume of a saturated neutralized solution of the salt. Additional solid ammonium sulfate was added to saturation. The resulting suspension was neutralized and refrigerated overnight. The sediment was collected by centrifugation on the following day, washed with a saturated solution of ammonium sulfate, and dissolved in distilled water. This solution was dialyzed against running water overnight, in cellophane tubing of 1/4" diameter. The supernate was similarly dialyzed. The volume of the redissolved sediment was then brought to the original volume of the solution of CSA, and the redissolved sediment and supernate were tested for complement-fixing activity. The activity of the original solution was completely recovered in the ammonium sulfate sediment, and no activity (less than 3%) appeared in the supernate. Analyses for nitrogen in the case of 3 pools of the CSA as produced in the synthetic medium yielded a mean value of 13.6% N relative to dry weight.

3. *Relation of complement-fixing antigens with other streptococcal antigens.* a. *Hemolysin-O.* The question of the relationship of streptococcal hemolysin-O to the complement-fixing antigens in CSA was approached by using erythrocytes as adsorbents of hemolysin. A solution of CSA, which contained both complement-fixing and hemolytic

TABLE III. Comparison of Complement-Fixation Titers of Various Preparations of Streptococcal Culture Supernates vs. Rabbit Antiserum with Activities of 3 Enzymes in Those Preparations. All values refer to relative concentrations of respective reagents in solutions of culture supernate concentrates at 1 mg/cc.

Preparation	Strain	CF titer	Hyaluronidase titer	Streptokinase titer	Desoxyribonuclease titer	Ratios		
						CF:H	CF:S	D:CF
130B	5797	200	<4	16	2000	>50	12.5	10
100A	H105	24	6	24	800	4	1	33
H7	NY5	1	4	<1	64	.25	>1	64
134B	H44	400	4	64	8000	100	6.3	50
H pool	H44	.5	16	<1	96	.03	> .5	192
CS "	H44	400	256	500	6000	1.6	.8	15
C3	H44*	12	96	<1	6000	.13	>12	500
CS 217	RG†	48	1	<1	3000	48	>48	63
CS 224 A	RJ†	32	.5	<1	1500	64	>32	47
CS 225	EC†	24	1	<1	1500	24	>24	63
Range of ratios:						>1600-fold	>60-fold	50-fold

* Cultivated in the Chemostat, under constant chemical conditions.

† Fresh human isolation, from non-inflamed pharynx.

activity, was incubated with a suspension of sheep erythrocytes at 4°C, both reagents having been chilled before incubation. After 30 minutes of such incubation erythrocytes were removed by centrifugation in a refrigerated centrifuge, and the supernatant fluid was tested for complement-fixing and hemolytic activity. The results are shown in Table II. It can be seen, on comparison of parts A and B, that the concentration of hemolysin in this preparation has been reduced to less than 6% by the incubation with erythrocytes, whereas the complement-fixing activity of this preparation has been reduced very slightly, if at all. Comparison of the data in part C with that in part B indicates that adsorption of hemolysin to erythrocytes takes place only if the former is in the reduced form. The fact that the very slight loss of complement-fixing activity in preparation B is also found in C would suggest that this loss was not related to the adsorption of hemolysin.

b. *Hyaluronidase, desoxyribonuclease and streptokinase.* Preparations of CSA derived from various cultures were tested for hyaluronidase, desoxyribonuclease and streptokinase activity in simultaneous tests, to obtain data on the association of any of these enzymes with the complement-fixing material. The results of some of these tests are shown in Table III. For inclusion in this Table, preparations were chosen which showed a relatively wide range of ratios of measures of relative activity

between complement-fixation and the respective enzymes. Table III shows that the range of ratios of complement-fixation titers to hyaluronidase titers was from 0.03 to >50, and that of streptokinase to complement fixing titers was from 0.8 to >48. The range of ratios of desoxyribonuclease to complement-fixation values was from 10 to 500.

c. *Complement-fixing somatic streptococcal antigens.* In earlier studies the preparation and complement-fixation reactions were described of 2 antigens or groups of antigens derived from extracts of sonically-disrupted streptococcal cells. These were designated as CP (cytoplasmic particles) and S (supernate proteins)(8,9). To obtain data on possible relationships between complement-fixing materials derived in the present study from the culture supernate, and those obtained by disruption of the streptococcal cell, a number of human sera were included in simultaneous complement-fixation tests against all 3 antigenic preparations, each used at its optimal concentration. The results of some of these tests are shown in Table IV. Data are presented in the case of 10 sera which include a relatively wide range of ratios of antibody titers to the 3 antigenic preparations. The range of ratios of anti-CSA to anti-CP titers in respective sera was from less than 0.1 to 2, and the range of anti-CSA titers to anti-S titers was from 0.5 to greater than 16.

Discussion. 1. *Production of complement-*

TABLE IV. Complement-Fixation Titers of 10 Sera Tested with 3 Streptococcal Preparations, CSA and 2 Somatic Fractions, to Illustrate Ranges of Relative Titers.

Serum No.	Titers vs.:			Ratio of:	
	CS	CP	S	CS/CP	CS/S
103	96	512	128	.2	.7
3821	12	128	8	.1	1.3
3955	128	96	12	1.3	11
4479	16	256	32	.07	.5
5119	128	192	8	.7	16
5267	128	128	12	1	11
5308	32	96	32	.3	1
5417	64	64	8	1	8
5657	768	384	128	2	6
5717	8	128	8	.07	1

fixing antigens in streptococcal culture. The data presented above would indicate that the substances which give rise to complement fixation with the sera used, are produced by the streptococcal cells in culture. This is suggested by the low yield in dry weight of uninoculated medium concentrated by similar procedures, by the negative complement-fixation tests found with such concentrates of the uninoculated medium, and by the concomitant rise in concentration of complement-fixing antigens in culture supernate with the turbidity of the culture. This antigenic material is broadly reactive, at least within Group A of β -hemolytic streptococci, since cultures of strains of almost all types within this group produced varying amounts of it. That this material, or at least a large part of it, is protein, is suggested by the fact that it is completely precipitable by ammonium sulfate at 80% of saturation, and by the mean nitrogen value of 13.6%. 2. *Relation of streptococcal CSA to known streptococcal antigens.* The question of identity of this complement-fixing material or of any components of it with any of the known streptococcal antigens cannot be properly approached until separation of such components has been achieved. In the single case of the O-hemolysin it has been possible to adduce fairly direct evidence that this substance is not responsible for the fixation of complement found with CSA, or at least for a major portion of it, since the adsorption of hemolysin from the crude concentrate by erythrocytes was not accompanied by a decrease in complement-fixing activity. The data on relationship of complement-fixing sub-

stances in CSA with other known streptococcal antigens is quite indirect and can be considered only as suggestive. Comparison was made of the concentration of complement-fixing antibodies to CSA with those to somatic nucleoprotein fractions of streptococcal cells to obtain some evidence as to whether the CSA might be largely derived from such cell fractions by dissolution of bacterial cells in culture. The wide range of ratios of antibody titers shown in Table IV would suggest that the fixation of complement by any cellular nucleoprotein which might have been released into the culture medium, could not have accounted for a major portion of the complement fixation in the tests with CSA.

The other group of antigens, examined in part, is that of the extracellular enzymes which, like the CSA, are found in the culture supernatant, and which might as antigenic proteins fix complement with homologous antibodies. While a complete list of such antigens was not examined, in experiments such as those reported in Table III, the wide disparity among measurements of complement-fixing activity, on one hand, and any of the 3 enzymatic activities, on the other, makes it quite unlikely that any of these 3 enzymes are responsible for a major portion of the complement fixation observed. Although there are, of course, other known streptococcal extracellular enzyme-antigens, the number of immunologically distinct antigenic species which would appear to be present in streptococcal CSA according to the data presented in the following paper (at least 7) would render it quite unlikely that the other streptococcal extracellular enzymes could be identical with the respective antigens found in this material. It would appear highly likely, therefore, that an antigen or antigens of streptococcal origin, not identical with any of the known streptococcal antigens, are present in the concentrates of streptococcal culture supernates.

Summary. 1. Supernates of cultures of β -hemolytic streptococci were concentrated with respect to macromolecular material present. Such concentrates gave positive complement-fixation reactions with sera of rabbits which had been given courses of injections of living

streptococci, and with sera of human subjects. 2. Evidence was obtained that complement-fixing antigenic material in such preparations was not identical with the complement-fixing fractions obtained from streptococcal cells, and complement fixation was probably not due to any of a number of the extracellular enzyme-antigens which can be found in streptococcal culture supernates.

1. Paul, J. R., and other contributors, *The epidemiology of rheumatic fever and some of its public health aspects*, ed. 2, New York, Metropolitan Life Insurance Company, 1943.

2. Harris, T. N., *Am. J. Dis. Child.*, 1948, v76, 411.

3. McCarty, M., in *Rheumatic Fever, a symposium*, Thomas, L., ed., p136, University of Minnesota Press, Minneapolis, Minn., 1952.

4. Green, C. A., *J. Path. and Bact.*, 1941, v53, 223.

5. Kirby, W. M. M., *PROC. SOC. EXP. BIOL. AND MED.*, 1951, v78, 519.

6. Harris, T. N., and Harris, S., *J. Bacteriol.*, 1953, v66, 159.

7. Bernheimer, A. W., Gillman, W., Hottle, G. A., and Pappenheimer, A. M., Jr., *ibid.*, 1942, v43, 495.

8. Harris, T. N., *J. Exp. Med.*, 1948, v87, 41.

9. ———, *ibid.*, 1948, v87, 57.

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Gel-Precipitation of Streptococcal Culture Supernates with Sera of Patients With Rheumatic Fever and Streptococcal Infection.* (21933)

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In the literature dealing with antigenic substances produced by the hemolytic streptococcus in liquid cultures the emphasis has been almost exclusively on those antigens which have some biologic activity, such as toxicity or enzymatic function. In present studies the reactions of tissues of the rheumatic host to streptococcal antigens are explored. These studies are concerned not only with streptococcal antigens which have known enzymatic function, but also with those which are demonstrable only by their ability to give rise to antibody formation and to combine with those antibodies in mass reactions such as complement-fixation or precipitation. Production and concentration of macromolecular materials produced in streptococcal culture, and their reaction in complement-fixation tests with sera of injected rabbits and human subjects are described in the preceding paper (1). The complement fixation test does not,

however, distinguish between one or more immunologic systems in solution (except in quite special circumstances of wide difference of concentrations of reagents relative to their specific activity). For the purposes of these studies methods of greater resolution were necessary to examine the antigenic materials produced in streptococcal culture. Accordingly, such materials were studied by gel-precipitin tests against rabbit and human sera. During such studies differences were noted in the numbers of antibodies present in sera of various groups of human subjects, in sufficient concentration to be observed. These will be presented here.

Methods and materials. 1. *Antigenic material.* Streptococci of Lancefield group A (Type 4, Strain H₄₄) were cultivated for 18 hours in the synthetic medium described by Bernheimer *et al.* (2). Supernates of centrifugation of such cultures were concentrated by precipitation with ammonium sulfate (at 80% saturation) in the presence of a cellulose fiber filter aid, Solka Floc. The extract of the resulting sediment was collected, suitably

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dialyzed and desiccated from the frozen state. The dry material was weighed and solutions of this material were used as described below. This material will be referred to as streptococcal CSA (culture supernate antigens).

2. *Sera.* Rabbits were given injections of CSA in either of two forms: weekly injections of approximately 5 mg CSA precipitated with alum, or tri-weekly injections, each 2.5 mg of the material in solution. Sera were obtained after a 4-week initial course of such injections and then, after successive 2-week courses of such injections, separated by one-week intervals. Human sera were obtained from 3 sources. Sera of presumably normal human subjects were obtained from outdated refrigerated bloods through the courtesy of the Philadelphia Serum Exchange. Sera of patients with scarlet fever were obtained from patients with this disease at the Philadelphia General Hospital at the beginning and end of their 3-week period of hospitalization, through the courtesy of Dr. A. C. LaBocchetta. A later specimen was obtained from a number of these patients approximately 6 weeks after the onset of streptococcal infection. Sera of patients with rheumatic fever were obtained from patients with a definite diagnosis of active rheumatic infection who were studied in the wards of the Philadelphia General Hospital, the Hospital of the University of Pennsylvania and the Children's Hospital of Philadelphia. Sera were obtained at the beginning of hospitalization and at 2-month intervals thereafter. Sera of patients with inactive rheumatic heart disease were obtained from patients at the Rheumatic Fever Clinic of the Philadelphia General Hospital. These patients were known to have had rheumatic fever at some earlier time (at least 2 years earlier) from study in the wards of above hospitals. 3. *The gel-precipitin tests. Preparation of agar.* Agar was prepared by immersing commercially available agar (Difco) in several volumes of cold distilled water, and incubating at refrigerator temperature. After the agar had settled, the water was decanted and the sediment resuspended in fresh water. After 5 such changes, swelling of the agar began and the washing was terminated. The sediment was now collected by centrifugation

and lyophilized. It was now white in color.

Technic of the tests. The following 2 procedures were employed: Single diffusion in 1 dimension: The procedure was described by Oudin, in which antigens diffuse from an overlying solution into a serum-agar gel(3). Double diffusion in 1 dimension: This method, described by Oakley(4), was used with modifications suggested by Preer(5). The technic involves 3 layers: antigen solution, an agar solution, and serum, from above downwards. In this procedure the antigens and antibodies each diffuse into the agar column. Bands of turbidity occur in the agar where any given antigen-antibody system is within the zone of equivalence, with a sufficiently high concentration of the antibody to cause visible precipitation. Soft glass tubes, acid cleaned, of 3 mm internal diameter and 10 cm length, were sealed at one end, lined with 0.3% washed agar solution, described by Oudin(3), and marked on the outside at 2, 3, and 5 cm from the bottom. Serum was added to the tube to the 2 cm mark (0.13-0.14 cc of serum). Bubbles were removed if they formed. Agar, in 0.3% solution containing merthiolate 1:10,000, which had been heated and then maintained at 48-52°C, was added slowly over the serum layer up to the 3 cm mark. The tubes were allowed to stand 15-20 minutes at room temperature to ensure complete solidification of the agar. The antigen solution, generally used at optimum concentration of 3 mg/cc and containing 1:10,000 merthiolate, was added up to the 5 cm mark. The tubes were sealed with adhesive tape, placed in a glass jar or desiccator, and stored in a cabinet at room temperature. In the majority of sera tested, the maximum number of bands was present on the third day. The test was read in a darkened room with the tube held against a fluorescent back light. The number of bands was determined, and a brief physical description of each band noted. Three grades of relative density of bands could be readily differentiated: C—clear, distinct, dense band, with sharp boundaries; F—faint, distinct, band with sharp boundaries, but not dense; VF—very faint band with clear boundaries. These gradations of density reflect relative concentrations of the respec-

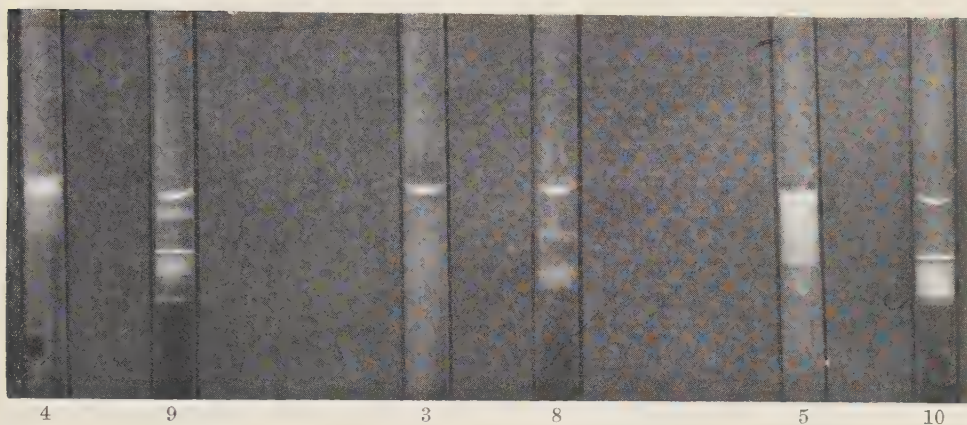


FIG. 1. Photograph of gel-precipitin tubes of 3 sera tested against concentrates of streptococcal culture supernate. Each pair of tubes is derived from a single serum, tested by single diffusion in the tube on the left and by double diffusion on the right. In all cases the meniscus constitutes the bottom of the antigen column. Note the greater resolution and the lower position (relative to the meniscus) of the bands in the case of the tubes set up by double diffusion (9,8,10).

tive antibodies at the level of the band, since the amount of specific precipitate reflects largely concentration of antibody. All bands not otherwise marked were of the dense type.

Fig. 1 shows gel-precipitin tubes of 3 sera tested by both single and double diffusion, on the third day of the test.

Results. 1. *Gel-precipitin tests with sera of rabbits injected with streptococcal CSA.* Rabbits given such courses of injections showed considerable variation in antibody response. Of those injected with alum-precipitated material only a few developed anti-CSA complement-fixation titers greater than 32, or more than 2 or 3 bands in the gel-precipitin tests, even after a number of courses of injection. Of the rabbits injected with the solution of CSA a higher percentage developed complement-fixation titers above 100 after the first 2 or 3 courses of injections, and at least 3 gel-precipitin bands within that time. Examination of the sera through the period of successive courses of injection with the CSA showed a general sequence of increasing complement-fixation titers and numbers of gel-precipitin bands against CSA from the pre-injection levels (CF titer below the threshold of measurement, and no gel-precipitin bands), although a number of instances of disparity occurred. Thus, one animal showed only 2 very faint bands and a complement-fixation

titer of 128 after the first course of injections, whereas another of that group at the same stage showed 3 bands by gel-precipitin with a complement-fixation titer of only 32. Of the entire group of animals, the best antibody response obtained corresponded to 5 bands in gel-precipitin tests and complement-fixation titers of 192-256.

2. *Tests with sera of presumably normal human adults.* Eighteen such sera were collected from the group of normal men. These subjects were approved as blood donors by recent medical history and superficial physical examination. Of 10 such sera chosen at random for gel-precipitin tests against streptococcal CSA, 2 showed 2 bands of precipitate, 7 showed 1 band and 1 showed none. Eight other sera were selected from a larger number on the basis of antistreptolysin and anti-hyaluronidase titers which were relatively high for the normal population (AH, 16-192; ASL, 64-256). Of these sera 1 showed 3 bands in the gel-precipitin test, 3 showed 2 bands, and 4 showed 1 band. No association was apparent between the level of the titers of the 2 neutralizing antibodies and the number of bands in the gel-precipitin test. Samples of 7 lots of gamma globulin prepared from pools of normal human sera, made available through the courtesy of the American Red Cross, were similarly tested. Of these.

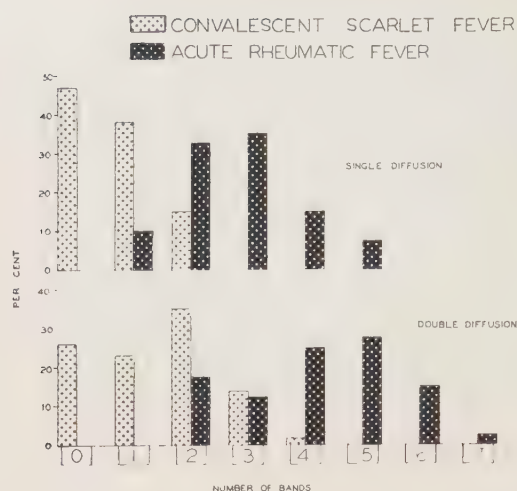


FIG. 2. Percentage distribution of sera of patients with acute rheumatic fever, and convalescent from scarlet fever, respectively, according to number of bands produced in tests with streptococcal culture supernates, by single-diffusion and double-diffusion techniques.

1 sample yielded 2 bands, 5 gave 1 band, and 1 gave none. These tests were done by the single-diffusion method.

3. *Sera of patients convalescent from scarlet fever and patients with acute rheumatic fever.* A group of sera obtained from patients convalescent from scarlet fever were tested for antibodies to antigens present in the streptococcal CSA. A total of 65 such sera were tested by the double-diffusion technic, 34 of which were also tested by single diffusion. The number of bands which appeared by the third day ranged from 0-2 in the case of the tests by single-diffusion and 0-4 by double diffusion. Sera which were obtained from 40 patients with acute rheumatic fever were also tested. Using the single diffusion technic the number of bands which developed ranged between 1 and 5, and in tests by double diffusion the number of bands ranged between 2 and 7. The sera tested in each group were distributed according to the number of bands which appeared by each method, and percentages calculated. Fig. 2 presents the data obtained. A larger percentage of sera from patients with acute rheumatic fever developed relatively greater number of bands within this distribution in tests, by both technics. Of one of the sera of a patient convalescent from scarlet

fever a larger specimen was available. Globulins were precipitated from a portion of this serum by adding an equal volume of a saturated solution of ammonium sulfate. The sediment was redissolved, and the solution dialyzed overnight and then brought to a volume equal to that of the original portion of serum. This solution yielded the same number of gel-precipitin bands as did the original serum.

4. *Sera of patients with scarlet fever at various times relative to the onset of the disease.* Of the sera of patients with scarlet fever a number were chosen whose convalescent sera had developed from 2-4 bands, for a comparison of the number of bands present early in the disease, at convalescence (3 weeks), and 6 weeks after the onset of symptoms. These were examined in a single test by double diffusion. The results are summarized in Table I. In all cases there was an increase in the number or intensity of the bands from the acute to the convalescent stage of this disease. In some cases, where bands were already present in the acute serum specimen, this difference was smaller. The relatively high antistreptolysin-O titers in some of these acute specimens would suggest, however, that some of these sera may have been obtained after the disease had been present long enough for some antibody response to have occurred. (It should be noted that the acute serum is obtained at the time of admission to the hospital, which is usually some days after the onset of symptoms. Also, the notation of day of disease on which the serum is obtained depended on the medical history provided by

TABLE I. Number of Gel-Precipitin Bands Produced by Sera from Patients with Scarlet Fever at Various Times in Relation to Onset of the Disease, Tested with Streptococcal CSA. (8 patients.)

Early serum specimen Day after onset of symptoms	Antistrep- tolysin titer	No. of bands		
		Acute	3-wk	6-wk
3	12	0	2	2 + 1F
3	256	1	2	2
4	64	0	4	1 + 2F
2	384	3F	3	2F
2	96	0	2	2
4	128	3F	3	2 + 1F
4	64	1VF	2	2
4	256	2	2 + 1F	3

TABLE II. Number of Gel-Precipitin Bands Produced by Sera of Patients with Acute Rheumatic Fever Observed over Periods Ranging from 4-18 Months. (18 patients.)

Acute serum	Months after onset					
	2	4	6	8	12	18
1 + 1F	3	2	2	1 + 1F	1 + 1F	1 + 1F
5F	1 + 1VF	1	1			1
4	2	1 + 1F	1 + 1VF	1F	1F	
5	1 + 1F	1F	1F	1VF	1VF	
3	2	2F			1 + 1F	
4	1 + 2F	2VF			1VF	
3	1 + 2F				1 + 1F	
7	1 + 1F	3F	2	1 + 1F		
5	1 + 1F	2	2	1 + 1VF		
3	2	1 + 1F	1F + 1VF	1F + 1VF		
5	2	3		2 + 1VF		
3	1 + 1F	1 + 1F	1			
3	2	2	2			
4 + 2VF	6	5	3 + 2VF			
6	3	2				
5	1 + 1VF	1				
6	3 + 3F	3				
5 + 1F	4F					

a parent.) In the sera obtained 6 weeks after the onset of symptoms no increase in the number of bands was found over the 3-week serum specimen, and occasionally a decrease in the number of bands was noted.

5. *Sera of patients with rheumatic fever at different stages of the disease.* A group of patients with rheumatic fever was studied over the period from acute disease to quiescence. For each patient, sera which were obtained in the acute stage and 2, 4, 6, 8, 12 and 18 months later were tested by double diffusion. There was a definite decrease in the number of bands, from the period of acute rheumatic fever to inactive rheumatic fever. In many instances some decrease in the number of bands was already evident 2 months after the acute attack. Table II summarizes the findings obtained in 18 patients with acute rheumatic fever followed for 4 to 18 months. Sera obtained in an out-patient clinic from 12 persons with inactive rheumatic fever were tested by the double-diffusion technic. Of these, 1 serum showed 3 bands; 3 sera 2 bands; 5 sera 1 band; and 1 serum no bands.

Discussion. Comparison of data obtained by single-diffusion (Oudin) and double-diffusion technics. In all groups of sera, both by single and double diffusion, the latter technic resulted in more bands, for the reason that in the double-diffusion method the concentration of any antibody varies over a continuous

range which extends to zero, whereas in the single-diffusion method the concentration of an antibody is never below a given fraction of its concentration in the serum.[†] A technical advantage of the double-diffusion technic is that it permits the use of turbid sera, or sera which have become turbid through freezing and thawing, since the observation of bands of precipitate is not made in the zone which contains serum. *Number of gel-precipitation bands obtained in various groups of sera tested with streptococcal culture supernate concentrates.* The data presented above indicate that there are a considerable number of antigenic components in concentrates of streptococcal culture supernates prepared for these studies. As many as 7 bands have been found in the case of single sera and the total number of antigenic species may be greater than that for 3 reasons. (1). Within any single tube the number of bands is a minimum

[†] In the case of single diffusion the concentration of antibody at the interface soon becomes one-half its concentration in the serum-agar column (i.e., one quarter its concentration in the original serum); concentration of antibody thus varies within the agar column from one-quarter to one-half its concentration in serum. In the case of the double-diffusion technic the concentration of any antibody within the agar column varies from zero, at the antigen interface, to one-half its concentration in the original serum, at the serum interface.

of the number of antigen-antibody systems present because the difference between rates of diffusion of 2 antigenic species may be too small for the limit of visual resolution of the corresponding bands; (2). identification of the bands with respective antigens is not possible at present, and we do not know that antigens from the same group of 7 are involved in all the bands observed in various sera; (3). there may be antigens to which the antibodies are not present in the rabbit or human sera in sufficient concentration to produce visible precipitate. Evidence that these bands are produced by precipitation of streptococcal antigens with homologous antibodies is offered by the fact that they appeared *de novo* in sera of rabbits injected with the streptococcal CSA, and by the time relation of numbers of bands found in the sera of patients with scarlet fever and rheumatic fever to the times of onset of the diseases.

The relationship of any of these antigens to the known extracellular enzymes of the hemolytic streptococcus is not known. There have been two reports of gel-precipitin studies of concentrates of streptococcal culture filtrates which had been partially purified with respect to a biologically active agent. Jennings(6) found 8 separate precipitin systems in preparations of streptococcal erythrogenic toxin tested by the Oudin technic against horse antitoxin, and identified one of these as the toxin itself. Recently Halbert *et al.* examined preparations of streptococcal hemolysin-O by using human and rabbit sera in the same technic and found as many as 4 bands. Here, again, evidence was presented that one of these was hemolysin, the other bands being due to other antigens, which had not been separated from the biologically active one for which partial purification had been made(7). Using a crude concentrate of streptococcal culture filtrate in which no separation of macromolecules had been attempted, these authors found as many as 7 bands in the sera of patients with acute rheumatic fever, and no more than 4 in sera of patients with miscellaneous diseases other than rheumatic fever (8). The data presented here on the numbers of bands obtainable in gel-precipitin tests between sera in acute rheumatic fever and crude

streptococcal culture supernate concentrates thus agrees with those of Halbert *et al.*

Difference between distributions of gel-precipitation band numbers in sera of patients with acute rheumatic fever and those convalescent from scarlet fever. The presence of a band in gel precipitation reflects the presence of an antibody in a concentration above the threshold required to produce a visible precipitate. Accordingly, the differences in band numbers found between these 2 groups of patients reflect higher concentrations of serum antibodies to streptococcal antigens in acute rheumatic fever than in convalescence from an uncomplicated streptococcal infection. It is not possible at present to compare antibody concentration to the individual antigens contained in these preparations, since there is no way of identifying the bands due to the respective antigen-antibody systems. This suggestion of higher concentrations of antibodies to streptococcal antigens in acute rheumatic fever, in comparison with streptococcal infection, agrees with observations by various workers in the case of a number of antibodies to streptococcal antigens (neutralizing antibodies to extracellular enzymes and complement-fixing antibodies to somatic nucleoproteins) (9,10).

No explanation can be suggested for the difference in range of distribution of serum antibody concentration to streptococcal antigens in these two diseases. Data are available on only one aspect of the contact between streptococcal antigens and the tissues of the rheumatic host which might result in such a difference—the greater interval of time than 3 weeks which might elapse between the onset of a foregoing streptococcal infection and admission to a hospital for rheumatic fever of many of these patients. That mere duration of contact between antigens and host tissues is not the causative factor is suggested by the fact that in the case of the individual patients with scarlet fever tested at both 3- and 6-week intervals after the onset of the disease there was no increase in the number or intensity of bands at the 6-week interval, in comparison with the 3-week, but rather a decrease in the intensity of some of these.

Summary. 1. Precipitin tests in semi-solid

agar (gel-precipitin) have been carried out between concentrates of streptococcal culture supernates and sera of various sources. Two technics of diffusion in 1 dimension (tube) were used: the single-diffusion technic of Oudin, and a double-diffusion technic. 2. Sera of rabbits previously injected with streptococcal culture supernates showed increasing numbers of bands as the course of injections progressed. Sera of such rabbits showed as many as 5 bands when tested with such concentrates, indicating that this was the minimum number of antigen-antibody systems present. 3. Sera of patients with acute rheumatic fever and of patients convalescent from an acute streptococcal infection. (scarlet fever) were also tested by this technic against concentrates of streptococcal culture supernates. The group with rheumatic fever showed a greater number of bands (a range of 2-7) than did the group with scarlet fever (0-4), when tested by the double-diffusion technic. The corresponding ranges found with the single-diffusion technic were 1-5 and 0-2,

respectively. In a sampling of sera from convalescents from scarlet fever the number of bands was found to be no greater 6 weeks after the onset of the infection than 3 weeks after the onset.

1. Harris, T. N., *Proc. Soc. Exp. Biol. and Med.*, 1955, v90, 33.
2. Bernheimer, A. W., Gillman, W., Hottle, G. A., and Pappenheimer, A. M., Jr., *J. Bacteriol.*, 1942, v43, 495.
3. Oudin, J., *Methods Med. Research*, 1952, v5, 335.
4. Oakley, C. L., and Fulthorpe, A. J., *J. Path. and Bact.*, 1953, v65, 49.
5. Preer, J. R., personal communication.
6. Jennings, R. K., *J. Immunol.*, 1953, v70, 181.
7. Halbert, S. P., Swick, L., and Sonn, C., *J. Exp. Med.*, 1955, v101, 539.
8. ———, *ibid.*, 1955, v101, 557.
9. Harris, T. N., Harris, S., and Nagle, R. L., *Pediatrics*, 1949, v3, 482.
10. McCarty, M., in *Rheumatic Fever, a symposium*, Thomas, L., ed., pp 135-149, University of Minnesota Press, Minneapolis, 1952.

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Teratogenic Effects of *Lathyrus odoratus* Seeds on Development and Regeneration of Vertebrate Limbs.* (21934)

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Diets containing *Lathyrus odoratus* seeds fed to young rats produce characteristic defects of mesodermal structures(1). Chemical analysis of extracts from the seeds points to the β -aminopropionitrile (BAPN) as the active fraction of the teratogenic principle(2-5). However other aminonitriles(5) and β -mercaptoethylamine(6) when given to rats have been reported to produce similar lesions. The *Lathyrus* poisons gain in interest through the similarity of the induced lesions with certain skeletal and vascular diseases in man, such as slipped epiphyses, degenerative arthritis, and dissecting aneurism of the aorta. Therefore

the use of aminonitriles in animal research seemed to offer an opportunity for the elucidation of these human diseases.

We reported(7) that BAPN at a concentration of 2 μ l/liter of aquarium water produces characteristic deformities in *Xenopus* larvae. The high sensitivity of *Xenopus* larvae suggests their use for the detection and quantitative evaluation of lathyrism-inducing agents. The effect of BAPN appears to be highly specific, affecting particularly certain rapidly developing mesodermal tissues. In view of the similarity between the processes of embryogenesis and of regeneration, the studies were extended also on the restitution of amputated legs in the newt, *Diemictylus viridescens*.

Methods. In a first series of experiments

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TABLE I. Effects Produced by Lathyrus Seeds, β -aminopropionitrile, and Other Compounds in *Xenopus* Larvae (Witschi's Stage 28). Class 1-4 express degrees of severity, 0 indicates normal development.

Compound	Conc., μ l/liter	No. of animals	Class
Powdered seeds*		20	2
Sweet pea water extract†		20	2
Acetonitrile	20	10	0
Propionitrile	20	10	0
α -aminopropionitrile	10	10	0
β -aminopropionitrile	10	20	4
	2	20	3
	1	20	2
	.5	20	1
	.2	20	0
Aminoacetonitrile (hydro-sulfate)	10	10	0
γ -aminobutyronitrile	10	10	0
Acrylonitrile	10	10	0
Glutamine	20	10	0
Potassium cyanide	10	10	0
Controls		100	0

* About 1 g of powdered seeds per day was placed in each aquarium; larvae were given no other food.

† Extract of 2.5 g of dry seeds per liter of water.

larvae of the South African toad, *Xenopus laevis*, were exposed to the natural principle by feeding powdered Lathyrus seeds *ad lib.* or weak solutions of BAPN,[†] or solutions of other related compounds. BAPN is a liquid and mixes with water freely; therefore concentrations are expressed in μ l per liter of aquarium water. Since preliminary experiments had shown that treatments of early stages are without effect on limb development, the here reported experiments were all started with nearly fullgrown larvae, 10 to 12 days before the expected breakthrough of the forelimbs. At this stage the legs are still short and not yet movable. Lots of 10 to 20 larvae were placed in aquaria containing 5 liters of dechlorinated water. In the *second series* of experiments, hen's eggs (White Rock breed) were injected with 20, 1, .2 or .1 μ l of BAPN in saline. Penicillin was added at the rate of 100 units per injection. The injections were made into the yolk sac either on the fourth or the tenth day of incubation, and the eggs were candled to observe the embryos daily

[†] Supplied through the courtesy of Dr. S. Wawzonek, who synthesized the compound from acrylonitrile and ammonia.

thereafter. In the *third series* of experiments, a hind limb of adult newts was amputated at the middle of the thigh. Immediately after the wound had healed 10 animals were placed in water containing BAPN at a concentration of 2 μ l/liter; and 10 served as controls.

Results. A. Experiments with Xenopus larvae (Table I). BAPN in concentration of 10 μ l/liter causes death of the animals during metamorphosis. The limb deformities are most severe and frequently subcutaneous hemorrhages occur in this group. The larvae exposed to lower effective concentrations complete their metamorphosis, but they are too weak to survive beyond this stage. Fig. 1 represents a metamorphosing tadpole from a lot receiving BAPN at 2 μ l/liter together with a control. The legs of tadpoles treated with this concentration are so badly twisted that it is impossible to prepare longitudinal sections for histological studies. At all levels of concentration the dislocation of the knee and ankle joints represents a most characteristic deformity (Fig. 2). A loosening of leg muscles, tendons and ligaments is clearly noticeable under a binocular microscope after removal of the skin. The microscopic examination of animals treated only 5 to 6 days shows wide clefts between cartilage and the covering connective tissue in the region of the joints (Fig. 3). In control animals of corresponding stage a compact embryonic connective tissue surrounds the cartilages and connects adjoining epiphyseal ends (Fig. 4). Obviously this rent formation is the beginning of the lesions in the young toads, a process which eventually results in dislocation of the joints. The cartilage appears normal and the periosteal ossification seems unaffected.

Lesions also develop in the dorsal musculature about 10 days following the onset of the experiments. After removal of the dorsal skin of tadpoles treated with BAPN or with Lathyrus seeds one observes that the intersegmental septa are abnormally wide (Fig. 1a, b). Detailed microscopic examination shows a very loose connective tissue in the septa. The muscle segments are atrophic.

The general body growth of the treated larvae becomes increasingly retarded. Experimental animals are thinner and weaker

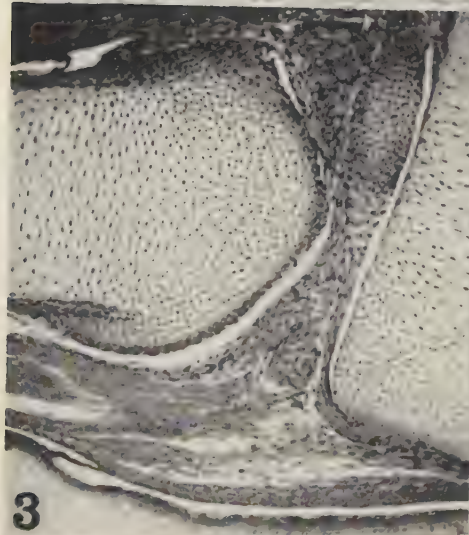


FIG. 1. a. *Xenopus* larva treated for 12 days with BAPN at a concentration of $2 \mu\text{l/liter}$. Skin removed, showing dorsal musculature and widened intersegmental septa. b. Normal control of same age, skin removed.

FIG. 2. Longitudinal section through a hind leg of a *Xenopus* larva treated with BAPN for 10 days; concentration: $1 \mu\text{l/liter}$; $\times 12$.

FIG. 3. Section through the knee joint of a young tadpole, stage 29, fed with sweet pea seeds, showing the presence of clefts between the connective tissue and the epiphysis; $\times 120$.

FIG. 4. Section through the knee joint of a control, stage 29; $\times 120$.

TABLE II. Teratogenic Effects of BAPN on Chick Embryos.

Dosage, μ l/egg	No. of eggs	No. died	Survived	Effect
20	13	13	0	Lethal
1	24	24	0	"
.2	20	13	7	Limb lesion
.1	10	6	4	" "
Control*	16	4	12	0

* Saline and penicillin.

than the controls, probably because food intake is impaired. The minimal dose producing noticeable effects is less than $.5 \mu$ l/liter. It is noteworthy that within each dosage group all animals, without exception, were affected similarly.

It is of interest that treatments started after metamorphosis remain ineffective even if continued over periods of 2 months. Only slight deformity of the hind-legs developed in a few animals that had been treated over a period of 3 months with a high concentration of BAPN (4μ l/liter). However, no dislocation of joints ever occurred.

All compounds other than BAPN so far tested gave negative results. The high potency of aminoacetonitrile in producing skeletal lesions in rats as reported by Wawzonek *et al.*(5) could not be verified in tadpoles.

Injections of α -tocopherol (total dose: 50 mg per tadpole) into the body cavity of tadpoles treated with BAPN did not protect the animals from developing the lesions. This result differs from that reported by Lee for the rats(8).

B. Deformities produced in chick embryos. In order to test the effect of BAPN on embryos of warmblooded animals, incubating hen's eggs were injected with BAPN. Since the embryonic development of the chick takes place within a closed egg shell, permitting no elimination, quantitative assays may be expected to give results of highest accuracy. The experiments are summarized in Table II. BAPN has a lethal effect at dosage levels of 1μ l and more per egg. The lesions produced in lower dosage groups are highly specific, affecting particularly the development of the legs (Fig. 5, 6). Size and weight of the animals are the same as those of the controls. Whether injected on the fourth or the tenth day, a bending of the femur end of the primary ossification center appears on the thirteenth day of incubation (Fig. 6). No dislocation of joints was observed. Cartilage and bones are histologically normal.

C. Deformities in regenerating limbs. Newts were used for this investigation because



FIG. 5. Chick embryo injected with $.2 \mu$ l BAPN; age 17 days of incubation.



FIG. 6. Longitudinal section through a leg of a 13-day chick embryo injected with $.2 \mu$ l BAPN; $\times 15$.



FIG. 7. Newt treated with BAPN during process of leg regeneration; concentration: 2 μ l/liter. Left side intact limb, normal; right side, regenerated limb, showing dislocation of joint; $\times 2$.

FIG. 8. Longitudinal section through regenerated limb in above figure. Note severe displacement at joint and atrophy of muscle; $\times 10$.

FIG. 9. Longitudinal section through a normal regenerated limb; $\times 10$.

of their well-known capacity of regeneration. After amputation of a hind limb it regenerates almost completely within about 3 months. The results of BAPN treatments are in principle as in the foregoing experimental series (Fig. 7). Microscopically the bones appear dislocated (Fig. 8, 9). Studies of serial sections also indicate atrophy of the leg muscles and loosening of the tendinous insertions.

Discussion. The results of the present series of studies indicate that BAPN is the teratogenic agent of *Lathyrus* seeds. In earlier experiments it was observed that 10 μ l/liter of BAPN did not visibly affect cleavage and gastrulation of *Xenopus* eggs. However, the dorsal musculature and the skin of the hatched larvae were severely damaged. Further studies on the effect of myotome development are in progress. At the present it may be concluded that BAPN affects a definitely limited stage of the process of muscle and tendon differentiation. The action of this compound is tissue specific, affecting mainly the mesodermal tissues, probably the intercellular ground substance; but it is not species specific, producing similar lesions probably in all vertebrates. The severe teratogenic effect on embryonic and regenerating tissues suggests that the formation or normal activity of

some essential morphogenic agent is inhibited by the presence of this compound.

Attempts at comparing the value of amphibians and of birds as bioassay animals are made difficult by the difference in the mode of administration of the compound. The chick as well as the amphibians are highly responsive and might be used for assay purposes in clinical work.

Summary. The evidence presented indicates that β -aminopropionitrile is the teratogenic agent of the seeds of *Lathyrus odoratus*. It produces dislocation of joints and loosening of intersegmental septa in amphibians, and bending of the femurs in chick embryos. The minimal teratogenic concentration for *Xenopus* larvae is .5 μ l/liter, the minimal dose for chick embryos is .1 μ l per egg. The effects are highly tissue specific but not species specific. The available evidence suggests that a single morphogenic process is interfered with by the presence of this compound during differentiation.

1. Ponseti, I. V., and Shepard, R. S., *J. Bone and Joint Surgery*, 1954, v36A, 1031.
2. Dupuy, H. P., and Lee, J. G., *J. Pharm. Assn, Sci. Ed.*, 1954, v43, 61.
3. Schilling, E. D., and Strong, F. M., *J. Am. Chem.*

Soc., 1954, v76, 2848.

4. Dasler, W., *Science*, 1954, v120, 307.

5. Wawzonek, S., Ponseti, I. V., Shepard, R. S., and Wiedenmann, L. G., *ibid.*, 1955, v121, 63.

6. Dasler, W., *PROC. SOC. EXP. BIOL. AND MED.* 1955, v88, 196.

7. Chang, C. Y., Witschi, E., and Ponseti, I. V., *Anat. Rec.*, 1954, v120, 816.

8. Lee, J. G., *J. Nutrition*, 1950, v40, 587.

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Histochemical Studies of Effects of Cortisone on Fetal and Newborn Rats.*† (21935)

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There is a growing body of experimental evidence demonstrating the effect of cortisone on enzyme systems(1-3). These investigations, for the most part biochemical in nature, have been carried out on a variety of cellular enzymes(4-6). Much emphasis has been placed on the effect of cortisone on alkaline phosphatase. A number of workers(7), utilizing histochemical methods, have observed an increase in the alkaline phosphatase content of the brush border of the kidney tubule after injection with cortisone. Moog(8-10), obtained a precocious increase of intestinal phosphatase upon injection of fetal or newborn mice with cortisone. She interprets these results as indicating that cortisone influences the basic metabolic processes which are responsible for phosphatase synthesis.

Ribonucleic acid (RNA) has also received much attention as a substance involved in cortisone action. Decreases in hepatic RNA after cortisone injection have been observed by several investigators(11), although there is some conflict on this point(12,13). One

group(7) noted an increase in renal tubule RNA after cortisone injection.

In view of the widespread interest in the mode of action of cortisone, it was decided to extend and correlate the above-cited findings. Because of the importance of the functional relationships existing between alkaline phosphatase, RNA and polysaccharide-protein complexes, histochemical methods were employed to follow the changes induced by cortisone on these materials.

Materials and Methods. Two series of animals were employed. The first series consisted of 24 female Wistar rats. Each female was placed with a male and daily vaginal smears were made until fertilization occurred (as evidenced by sperm in the smear). Beginning with the seventh day of pregnancy, the mothers were divided into 3 equal groups. Group I animals received 75 mg of cortisone per kilo of body weight daily throughout pregnancy; Group II animals received 100 mg and Group III served as controls. The cortisone was divided into 2 daily doses, given 8 hours apart.

Each of the above groups was subdivided into 2 sub-groups of equal size. In one Caesarian sections were performed on the fourteenth day and in the other on the sixteenth day of gestation. The uterine horns were exposed under moderate nembutal anesthesia, a tally of the number of fetuses present was taken and one-half of the number was removed. The incisions were then closed and

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† Cortisone acetate (Cortone, Merck) used for these experiments was generously supplied by Merck and Co., Rahway, N. J.

‡ Based in part upon a dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at New York University.

cortisone treatment was continued. A second Caesarian section was performed 4 days after the first, in each case yielding 18 and 20 day fetuses respectively. All fetuses were weighed on a torsion balance and fixed *in toto* at 4°C for 24 hours in Bouin's, Carnoy's or 95% ethyl alcohol. A total of 201 fetuses was so obtained.

In the second series of experiments, a total of 110 post-partum rats was used. Litters were kept with the mother and beginning with 24 hours after the hour of birth, the litter mates were divided into 2 groups. The first group received a daily injection of 0.1 mg of cortisone while the second group served as vehicle-injected controls. Injections continued until 24 hours before autopsy. At least 2 complete litters were autopsied on each of the following days of age: 3, 5, 7, 9, 11, 13, and 15. Liver, intestine and kidney were fixed as in the first series.

In addition to a routine hematoxylin and eosin stain, the following histochemical methods were employed. Alkaline phosphatase was demonstrated by the Ca-Co method of Gomori(14). Both DNA and RNA were visualized by methyl green-pyronin Y used according to the methods of Taft(15) and Kurnick(16). To substantiate the specificity of the stain, alternate sections were digested with a solution of ribonuclease (Worthington) in distilled water. Mucopolysaccharides were identified by the periodic acid-Schiff method of McManus(17). Glycogen was identified by malt diastase digestion.

Results. As was to be expected, at all ages the cortisone-injected animals showed a significant inhibition of weight gain as compared to the controls. This was evidenced by a marked smallness in size of the fetal rats and by a general debilitated appearance in the post-partum rats. There was no difference in response between the fetal rats whose mothers received the 75 mg dosage and those of mothers receiving the 100 mg dosage.

Fetal Rats. In the fetal rats, beginning with the 16th day of intra-uterine life, the hepatic parenchyma of the cortisone injected animals showed increases in glycogen deposition and decreases in RNA content when compared with controls of the same age. This

has been a constant finding in all previous studies in this area(11-13). No differences became observable in the intestinal epithelium and renal tubule epithelium of the hormone-treated animals until the 16th day of intra-uterine life. At this age, there is the beginning of a response to hormone treatment which becomes accentuated at 18 days of age. By the 18th day, there is a higher RNA content in the supra-nuclear regions of the intestinal epithelium in the hormone-treated fetuses than in the controls of the same age. This increase of RNA is even more pronounced at 20 days of age, where the controls show only a moderate RNA content (Fig. 1a) while the experimentals demonstrate quite marked accumulations of RNA (Fig. 1b).

Similarly, in the brush border of the renal tubules there is an increased deposition of RNA in the cortisone-treated animals. This increase begins at 16 days of age and is quite pronounced at 18 days; the controls showing only a moderate RNA brush border content (Fig. 2a) while the treated animals demonstrate heavy deposits in the equivalent loci (Fig. 2b). This difference is no longer evident at 20 days of age.

As far as PAS-positive materials are concerned, the only significant differences are found in the renal tubule epithelium of the 18-day-old fetuses. In this site, there is evidence of increased glycogen deposition in the treated fetus (Fig. 3b) as compared with the control (Fig. 3a).

Along with these increases in RNA, concomitant increases in alkaline phosphatase were evident. Signs of increased alkaline phosphatase activity of the striated border could be seen in the 18-day-old treated fetuses. This heightened enzyme activity in the cortisone injected animal was more outstanding at 20 days of age. In the control animal of this age, the intestinal striated border shows only a slight alkaline phosphatase activity (Fig. 4a). The hormone treated animal, on the other hand, exhibits a marked activity which extends into the supra-nuclear cytoplasm (Fig. 4b). Both of these slides were incubated in the substrate for 10 minutes.

In the renal tubule epithelium, the cortisone treated animals exhibit an increased alkaline

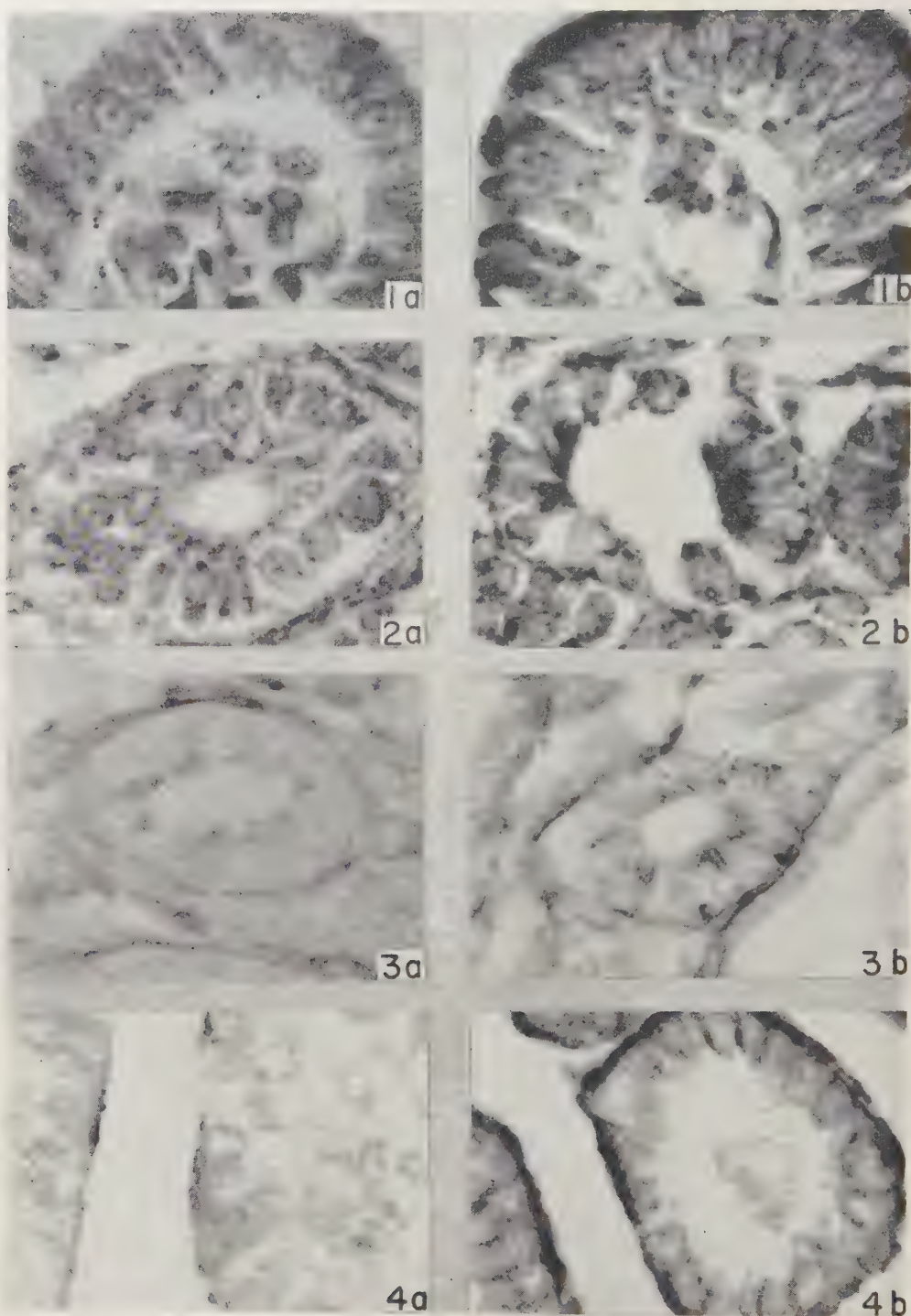


FIG. 1. Intestinal epithelium, 20-day-old fetal rat. Methyl green-pyronin. a, control; b, experimental. Note marked deposition of supra-nuclear RNA in treated fetus. $\times 600$.

FIG. 2. Renal tubule, 18-day-old fetal rat. Methyl green-pyronin. a, control; b, experimental. Note increase in brush border RNA in b. $\times 600$.

FIG. 3. Renal tubule, 18-day-old fetal rat. PAS. a, control; b, experimental. Note deposition of glycogen in and adjacent to brush border in b. $\times 600$.

FIG. 4. Intestinal epithelium, 20-day-old fetal rat. Alkaline phosphatase, ten min. incubation time. a, control; b, experimental. Note in treated fetus (b) greater alkaline phosphatase activity as compared to control (a). $\times 600$.

phosphatase content beginning with 16 days of age. This difference is even more exaggerated at 18 days. At this time, the controls show very little brush border alkaline phosphatase activity (Fig. 5a). The treated animals, on the other hand, demonstrate a very marked enzyme activity in the same site (Fig. 5b).

Post-partum Rats. In the post-partum rats, the cortisone injected animals at all ages exhibited a marked increase in hepatic glycogen deposition and a decrease of RNA content. As far as RNA is concerned, while the controls showed heavy deposits of RNA, usually in the form of rodlets or spheroids (Fig. 6a), the experimental animals demonstrated only scattered remnants of this material (Fig. 6b).

As far as the intestine and kidney are concerned, no effects of cortisone treatment were observed until the 9th day of age. At this time, significant increases in alkaline phosphatase appeared in the treated animals. This became more marked in the 1-day-olds (the oldest age group that survived the cortisone treatment). There were no such corresponding increases in RNA and PAS-positive materials. The intestinal epithelium of the 11-day-old control animals showed a moderate striated border alkaline phosphatase activity (Fig. 7a). Treated animals of the same age, on the other hand, show a more marked activity in the same site (Fig. 7b). The renal tubule brush border activity is similarly enhanced, the controls showing only moderate alkaline phosphatase deposition (Fig. 8a), while the experimental animals demonstrate a strong activity (Fig. 8b).

Discussion. Essentially, the results herein presented are in accord with the findings of Karnofsky and his group (18) on the injection of cortisone into the chick embryo and with Moog's (6-8) experiments on the influence of cortisone on the fetal and post-partum mouse.

In our work, we have observed that there were no histochemically demonstrable effects of exogenous cortisone administration in the

fetus until the 16th day of intra-uterine life. Karnofsky and his group noted that the chick embryo did not show a significant response to cortisone administration until after the 10th day of development. They maintained that the cortisone, in its observable effects, influences the utilization of a new substrate which is provided for cell growth as a result of the shift in embryonic metabolism occurring at this stage. The same line of reasoning might be applied to the present work on the rat, wherein a sensitive indicator of cortisone action, the liver, showed no response until the 16th day of fetal life.

Our observations have indicated that the RNA, alkaline phosphatase and glycogen of the renal tubule brush border are increased in the treated fetus as compared with the controls beginning with the 16th day and that this difference is no longer in evidence by the 20th day. The striated border of the intestinal epithelium of the treated animals did not show any increases in alkaline phosphatase and RNA until the 18th day. This increased response, however, was obtained through the 20th day. In both the renal tubule and intestinal epithelium the cortisone served to bring about a precocious acquisition of RNA.

In the post-partum rats a similar picture was observed. A response of the intestinal and renal tubule epithelium to cortisone could not be demonstrated in rats younger than 9 days of age. As in the fetuses, the response was exhibited as a precocious increase in the amounts of alkaline phosphatase and RNA. Moog has suggested that the intestinal epithelium passes through a transitory state of sensitivity to cortisone as a stage in its normal developmental sequence. The addition of excess exogenous cortisone merely hastens the basic metabolic response processes involved in the functional differentiation of both the intestinal and renal tubule epithelium.

Thus, there would appear to be 2 stages of sensitivity to cortisone in the rat. The first

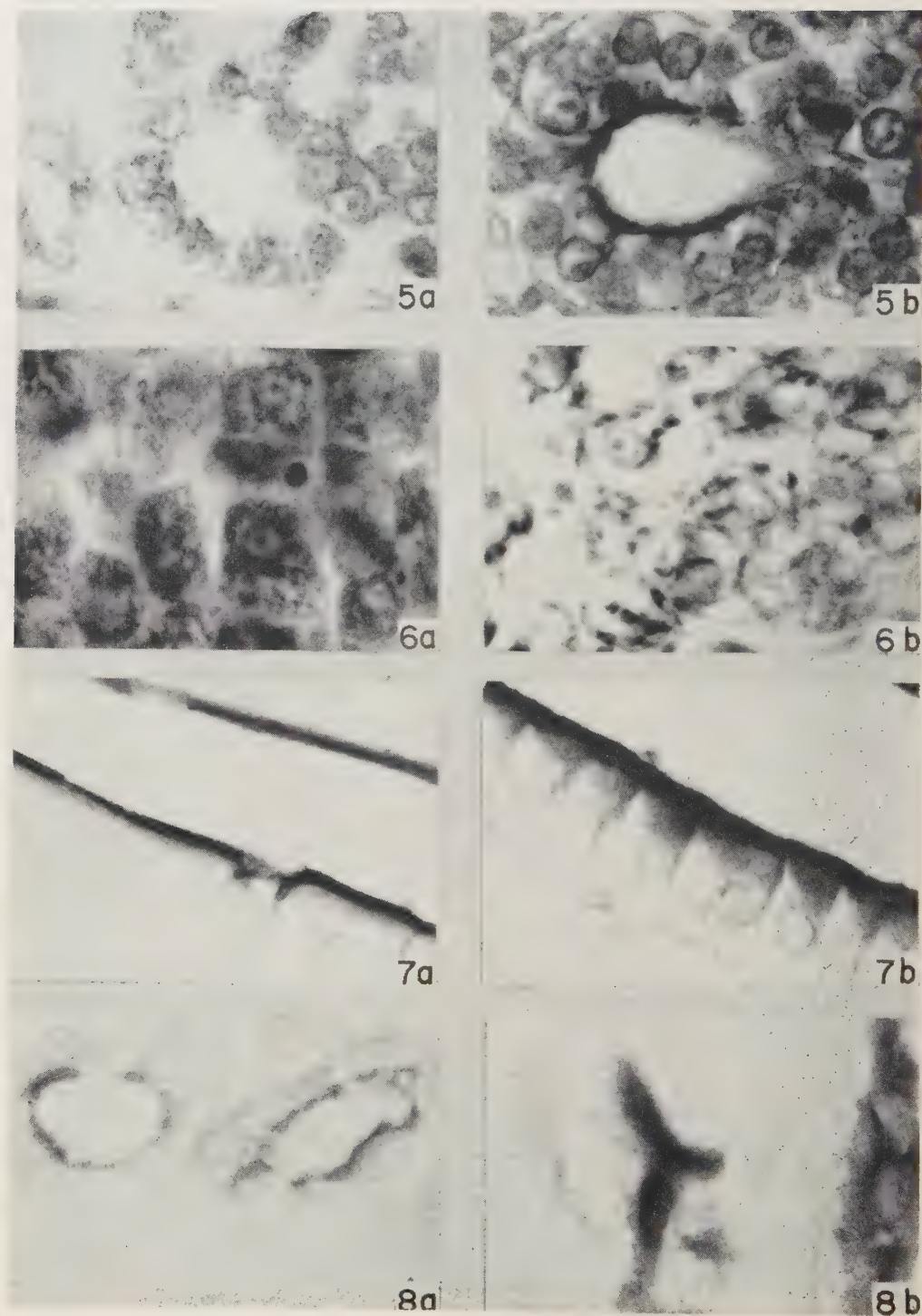


FIG. 5. Renal tubule, 18-day-old fetal rat. Alkaline phosphatase, 10 min. incubation time. a, control; b, experimental. Note very strong enzyme reaction in treated fetus (b) and contrast this with control (a). $\times 600$.

FIG. 6. Liver, 11-day-old post-partum rat. Methyl green-pyronin. a, control; b, experimental. Marked depletion of RNA is very prominent in b. $\times 600$.

FIG. 7. Intestinal epithelium, 11-day-old post-partum rat. Alkaline phosphatase. Ten min. incubation time. a, control; b, experimental. Treated rat (b) shows a strong enzyme reaction evident even in supra-nuclear cytoplasm. $\times 600$.

FIG. 8. Renal tubule, 11-day-old post-partum rat. Alkaline phosphatase, 10 min. incubation time. a, control; b, experimental. Note greater brush border phosphatase activity in b. $\times 600$.

appears in the embryo at 16 days of fetal life for the kidney tubule and at 18 days for the intestinal epithelium. The former is lost by the 18th day, whereas, the latter is still evident at 20 days. In the post-partum rat, the stage of sensitivity first appears at 9 days of age for both kidney and intestinal epithelia.

Baker and Bridgman(19), who studied the gastric and intestinal mucosa in adrenalectomized adult rats, noted a decrease in cytoplasmic RNA and striated border alkaline phosphatase activity. Following the injection of cortisone into these animals the RNA was reconstituted but the amount of alkaline phosphatase present was not affected. These findings lend further support to our observations as well as those of Moog(9) that after a certain stage of development cortisone is apparently no longer able to influence the formation of alkaline phosphatase in the intact animal.

It should, however, be emphasized that had Baker and Bridgman used whole adrenocortical extract, they might have altered the alkaline phosphatase activity in the adrenalectomized animal.

Summary. A histochemical study of the effect of cortisone on the liver, intestine and kidney of fetal and post-partum rats was made. There were no histochemically observable effects of cortisone on the fetus until the 16th day of age. Beginning with this day it was found that cortisone caused an increase in glycogen deposition and a decrease in RNA content of the hepatic parenchyma. In the post-partum rats these liver effects were noted at all ages. The intestinal striated border demonstrated a precocious acquisition of alkaline phosphatase and RNA as compared to

controls of the same age. In the renal tubules these effects were manifest in the 16- and 18-day-old fetuses, while the intestinal effects were obtained in the 18- and 20-day-old fetuses. Similar precocious increases in renal and intestinal alkaline phosphatase and RNA were noted in the cortisone-treated newborns beginning with the ninth day of age.

1. Soicher, R. R., *McGill Med. J.*, 1951, v20, 191.
2. Umbreit, W. W., *Ann. N. Y. Acad. Sci.*, 1951; v54, 569.
3. Verzar, F., *Vitamins and Hormones*, 1952, v10, 297.
4. Kochakian, C. D., and Vail, V. N., *J. Biol. Chem.*, 1944, v156, 779.
5. Dorfman, R. I., *Vitamins and Hormones*, 1952, v10, 331.
6. Kerpolla, W., *Endocrin.*, 1952, v51, 192.
7. Herlant, M., and Timiras, P. S., *Acta Anat.*, 1952, v14, 65.
8. Moog, F., *J. Exp. Zool.*, 1951, v118, 187.
9. ———, *ibid.*, 1953, v124, 329.
10. Moog, F., and Thomas, E. R., *Endocrin.*, 1955, v56, 187.
11. Williams, W. L., *et al.*, *Anat. Rec.*, 1953, v115, 247.
12. Fraenkel-Conrat, J., and Li, C. H., *Endocrin.*, 1949, v44, 481.
13. Gros, F., Bonfils, S., and Macheboeuf, M., *Compt. Rend. Acad. Sci.*, 1951, v233, 990.
14. Gomori, G., *Proc. Soc. Exp. Biol. and Med.*, 1939, v42, 23.
15. Taft, E. B., *J. Exp. Cell Res.*, 1951, v2, 312.
16. Kurnick, N. B., *Stain Tech.*, 1952, v27, 233.
17. McManus, J. F. A., *ibid.*, 1948, v23, 99.
18. Karnofsky, D. A., Ridgway, L. P., and Patterson, P. A., *Endocrin.*, 1951, v48, 596.
19. Baker, B. L., and Bridgman, R. M., *Am. J. Anat.*, 1954, v94, 363.

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Electrolyte Balances Following Total Body X-Irradiation. (21936)

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One of the prominent features of irradiation sickness is the attendant upset of salt and water metabolism. Depending upon the species, this may be manifested in the forms of vomiting, diarrhea, and diuresis. An exact determination of the changes in electrolyte balance following total body x-irradiation is, therefore, of interest.

Methods. Twenty 250 g male white rats (Rolfsmeyer Co., Madison, Wisconsin) were placed in metabolism cages over siliconized glass funnels, and allowed free access to distilled water and a diet containing 60% sucrose, 18% lactalbumin, 16.9% Crisco, 4% modified Wesson salt mixture (minus its Na⁺, K⁺, and Cl⁻), 1% liver extract (Wilson Co., liver fraction D, Lot 41863), 0.1% choline hydrochloride, with supplements of thiamine, pyridoxine, riboflavin and calcium pantothenate. Roughly 5% of the Na⁺, K⁺, and Cl⁻ intake was furnished by the diet; coming largely from the liver extract. The remainder of the sodium and potassium chlorides was administered daily by intraperitoneal injection. In the control period the average total electrolyte intake was 1.55 meq of sodium, 2.37 meq of potassium, and 2.54 meq of chloride per 48-hour period. Under these conditions the electrolyte intake was largely independent of the level of food intake. Each 48 hours, the food intake and body weight were recorded, and the excreta were removed for analysis. The excreta were collected quantitatively in barium hydroxide solution, evaporated to dryness, and ashed at 500°C for 12 hours. Sodium and potassium determinations were made on food, injection solutions, and excreta, with a flame photometer attachment to the Beckman DU Spectrophotometer. Chlorides were determined on the same samples by the Volhard method. After 18 days of control study, 16 of these animals were given a total body dose of 700 r of x-ray (140 kv., 0.25 mm Cu, 1 mm Al). In preliminary tests, with larger groups of animals, this dose was

TABLE I. Difference between Electrolyte Balances of Experimental and Control Rats before and after Total Body X-irradiation.

Pre-X-ray days	K ⁺	Na ⁺	Cl ⁻
0- 2	+ .050	+ .180	+ .124
2- 4	- .093	+ .145	+ .050
4- 6	- .081	+ .127	+ .014
6- 8	+ .156	+ .031	+ .047
8-10	- .113*	+ .061	+ .168
10-12	+ .162	+ .107	+ .214
12-14	+ .016	+ .196	- .077
14-16	- .100	- .121	- .007
16-18	- .136	- .008	- .364
Avg =	- .015	+ .080	+ .019
σ =	.116	.101	.169
Post-X-ray days			
0- 2	- .567†	- .245*	+ .066
2- 4	- .866†	- .558†	+ .124
4- 6	- .112	+ .045	- .424*
6- 8	- .033	- .178*	- .143
8-10	+ .214	+ .312	+ .183

* Significant at 5% level ($.01 < P \leq .05$).

† " " 1% " ($P \leq .01$).

found sufficient to kill half of the animals within a 2-week period. Balance studies were continued for an additional 10 days following irradiation. Four of the animals maintained in metabolism cages were used as parallel controls. They received no x-irradiation, but otherwise were handled in the same manner as the experimental animals. Balance data obtained from these controls were used as a base-line in the interpretation of the data obtained from the irradiated animals. The figures in Table I represent the difference in balance between the experimental and the control groups at each period. In this way it was possible to minimize any effect due to handling at the time of irradiation, and to compensate for the fact that the 300 g male white rat was still gaining weight. This procedure also tends to minimize the effect of any consistent bias in analytical methodology.

Results. In Table I it is seen that the potassium, sodium, and chloride balances are significantly altered following total body x-irradiation with an LD₅₀ dose of x-ray. There

is a marked loss of potassium during the first 4 days following irradiation, with a gradual recovery to a positive value by 8-10 days following irradiation. The changes in sodium balance are roughly parallel with those in the potassium balance data. Quite a different picture is seen in the chloride data, however. The chloride balance remains close to zero for the first 4 days of the post-irradiation period, and only after the 4th day is there a significantly negative balance. Again, there is evidence of recovery in the later periods.

The data of Table I represent the means of over 800 independent determinations carried out in triplicate. The control day-to-day standard deviations, representing the over-all analytic and physiologic variations, are only 5-7% of the intake. This degree of uncertainty is even further decreased by the large amount of control data available for comparison. The changes observed under these conditions are statistically significant ($P < .05$ for chloride and $P < .01$ for sodium and potassium), and clearly define the nature of the post-irradiation electrolyte balance changes in the rat exposed to an LD_{50} dose of total body x-irradiation.

Discussion. From previous reports in the literature, it can be inferred that following irradiation the body electrolyte balances become positive(1-3), negative(4-7), or are uninfluenced(8). Unfortunately, none of these

reports presents balance results which are statistically significant.

The main features of the post-irradiation electrolyte balances, as defined in Table I, are: (A) a prompt (0-4 day) loss of K^+ and Na^+ , (B) a delayed (4-6 day) loss of Cl^- , and (C) a recovery of electrolyte beginning at the 8-10 day period.

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1. Bennett, L. R., Bennett, V. C., and Howland, J. W., *Fed. Proc.*, 1949, v8, 350.
2. Cameron, A. T., and McMillan, J. C., *Lancet*, 1924, v2, 365.
3. Cori, C. F., and Pucher, G. W., *Am. J. Roentg.*, 1923, v10, 738.
4. Bowers, J. Z., and Scott, K. G., *PROC. SOC. EXP. BIOL. AND MED.*, 1951, v78, 645.
5. Edelmann, A., *Fed. Proc.*, 1949, v8, 39.
6. Glenn, J. L., and Bass, W. R., *ibid.*, 1954, v13, 55.
7. Soberman, R. J., Keating, R. P., and Maxwell, R. D., *Am. J. Physiol.*, 1951, v164, 450.
8. Bowers, J. Z., Davenport, V., Christensen, N., and Goodner, C. J., *Radiology*, 1953, v61, 97.

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Studies on the O Antigen of *Salmonella typhosa* IV. Endotoxic Properties of the Purified Antigen. (21937)

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Toxic manifestations induced in man and experimental animals by Gram-negative bacteria have been established as referable to their production of a distinctive component, variously described as somatic antigen, endotoxin, pyrogen, and tumor hemorrhagic agent. These specific attributes of endotoxins have been described, particularly by Burrows(1), Bennett and Beeson(2), and Thomas(3). Endotoxins are generally referred to as com-

plexes of polysaccharide, lipid, and protein; however, the precise chemical composition of the complex in relation to endotoxic activity has not been established. Biological properties of endotoxins have been studied for the most part with relatively crude preparations, frequently culture filtrates or extracts(4). Those purified products in recent investigations(5-9), contained significant quantities of nitrogen, which probably represented appre-

ciable protein or polypeptide components. Thus, the essentiality of the protein moiety for biological activity remains obscure.

Our papers described isolation of the somatic antigen of *Salmonella typhosa* as a lipopolysaccharide containing only 0.6% nitrogen, and showed that despite the essentially complete removal of protein, its activity as an antigen was not diminished, but increased (10,11). This report shows that this product possesses the properties characteristic of the classical endotoxins. Inasmuch as the biological effects of endotoxins are well-documented, this report is restricted to a summary of data on endotoxic activities of the lipopolysaccharide derived from *S. typhosa*.

Materials and methods. Endotoxin: The product was isolated by Dr. M. E. Webster, from viable *S. typhosa*, 0-901, by an ethanol-ammonium sulfate fractionation procedure. The purified endotoxin is a phosphorylated lipopolysaccharide containing only 0.6% nitrogen. Its chemical and immunological properties have been described (10,11). **Pyrogenicity tests:** Female New Zealand white rabbits weighing approximately 3 kg were employed. Animals free of demonstrable typhoid O agglutinins were utilized. They were quartered in individual cages in animal room maintained at 75°F. Rectal temperatures were obtained with clinical mercury thermometers. Normal or baseline temperatures were obtained several days prior to use. Following administration of test material, temperatures were taken hourly for 5-7 hours. Rabbits were not used for more than a single test.

Results. General toxic properties: The purified lipopolysaccharide derived from *Salmonella typhosa* possessed, to a high degree, the toxic attributes of colonially smooth Gram-negative bacilli. When administered intravenously to susceptible host, such as the rabbit, exceedingly small amounts were capable of inducing fever, leucopenia, diarrhea, prostration, and death. The activity of the purified endotoxin in eliciting this toxic syndrome is as follows:

Lethal Activity: The lethal action of intravenously administered endotoxin for rabbits is shown in Table I. Despite control of

TABLE I. Lethality of Typhosa Endotoxin for Rabbits.*

μg endotoxin (i.v.)	No. of animals dead
	Total animals inj.
10	2/10
20	7/10
50	6/10
100	3/10
500	8/15

* New Zealand white females weighing 2.1-2.4 kg.

strain, sex, weight, and age of rabbits, considerable variation in susceptibility of individual animals to the lethal action of the endotoxin exists. It is significant that administration of endotoxin over a wide range of dosage in no instance resulted in 100% mortality. On several occasions, the percentage of rabbits succumbing to large doses of endotoxin (100 to 500 μg) was less than that obtained with 20-50 μg . At present there is no explanation for this paradox. Despite this apparent lack of dose-response relationship, a large series of individual tests in this and in related studies have shown that the lipopolysaccharide generally, but not invariably, is lethal for rabbits in dose range of 20-50 μg .

As observed by other workers (1), we found that in contrast to the rabbit, the mouse is relatively refractory to endotoxin. While much larger doses (on the basis of body weight) are required, the lethal action of intraperitoneally injected endotoxin in this species was more reproducible and consistent than in the rabbit. Thus, the LD₅₀ for 15 g Bagg albino mice was approximately 250 μg . Like the mouse, the guinea pig tolerated relatively large doses of endotoxin. The LD₅₀ for this species was somewhat more variable and lay in the range of 500-1000 μg .

The lethal activity of the lipopolysacchar-

TABLE II. Lethality of Typhosa Endotoxin for Mice. Effect of alkali treatment.

μg endotoxin (i.p.)	Endotoxin	Alkali-degraded endotoxin
200	7/10*	0/10
400	8/10	0/10
600	6/10	0/10
1000	10/10	0/10
3000	—	3/10

* Fractions equal No. of animals dead of total animals injected.

TABLE III. Pyrogenic Activity of Typhosa Endotoxin. Comparison with intact typhoid bacilli.

Preparation	Dose (μ g)	Pyrogenic response of individual rabbits ($^{\circ}$ F)				Mean temp. rise ($^{\circ}$ F)	M.P.D.* (μ g)
<i>S. typhosa</i> 0-901	.3	2.6	1.4	1.7		1.9	.015
	.03	2.0	1.1	.8		1.3	
	.003	.4	.7	.1		.4	
Purified typhosa endotoxin	.008	2.2	2.9	3.0	3.1	2.8	.0002
	.0016	1.3	2.1	1.6	1.9	1.7	
	.00032	1.5	1.3	1.2	1.1	1.2	
	.00006	.4	.6	.5	.3	.4	

* Minimum pyrogenic dose: Quantity giving rise to 1° F elevation in temperature.

ide was considerably decreased following autoclaving, boiling for 30 minutes, or exposure to weak alkaline hydrolysis (*i.e.* 0.02 N NaOH for 18 hours at room temperature). A comparison of the lethal action for mice of alkali-d₂-graded endotoxin with undegraded endotoxin is illustrated in Table II. On a weight basis, the product subjected to alkaline hydrolysis exhibited less than 10% of the lethal activity of the untreated product.

Pyrogenicity. During investigations on the chemical and immunological properties of the typhosa endotoxin, it was observed that intravenous injection of rabbits with 0.01 μ g or less of this lipopolysaccharide resulted in rapid production of fever, associated with leucopenia and subsequent leucocytosis. Generally, temperatures rose within an hour after administration of the product, reached a maximum at approximately 3 hours, and gradually declined to normal in 6 to 8 hours. A marked neutrophilic leucopenia was observed one hour after injection, and the total leucocyte count gradually rose to normal at 4-6 hours, followed by a leucocytosis. These alterations in temperature and leucocyte counts were primarily dependent on the dose of lipopolysaccharide administered. The remarkable pyrogenic activity of this lipopolysaccharide is shown in Table III where the response of groups of rabbits to graded doses of this product are compared with those of animals receiving acetone-killed and dried typhoid bacilli. When the mean temperature response is plotted against dose of lipopolysaccharide administered, the response is rather well related to dose over the range shown in this Table. It is apparent that the purified product is a far more potent pyrogen

than the whole organism. The quantity of this component present in intact typhoid bacilli of the 0-901 strain has been reported as 3.3% by Staub and Combes(12), and 6.2% by Webster *et al.*(10). These data indicate that the isolated component should be approximately 15-30 times as active as the bacterial cell. The finding that it is in fact somewhat more active suggests that greater availability may favorably influence pyrogenic activity.

A state of insusceptibility to the pyrogenic action of this endotoxin could be developed in rabbits by a series of injections of the lipopolysaccharide. A group of 10 rabbits treated with 10 daily injections ranging from 1-20 μ g (initial dosage 1 μ g was progressively increased at 2-day intervals), exhibited little or no thermal response to 1 μ g of endotoxin 24 hours after the last injection (mean temperature rise of 0.6° F). This is in contrast to the mean temperature rise of 4° F. developed following injection of 1 μ g on the first day of the experiment. On the other hand, a constant daily dose of 1 μ g administered to a second group of 6 rabbits for a period of 15 days, did not result in as significant a depression of the fever peak obtained after the initial injection. After 1, 2, 3, 10, and 15 injections, mean temperature rises were 4.6° , 3.5° , 2.9° , 2.6° and 2.4° respectively. In addition, this peak was attained relatively earlier as the injection series progressed.

Animal species were found to vary greatly in their pyrogenic response to this product. Thus, the administration of 10 μ g of lipopolysaccharide intravenously brought about a marked thermal response in 2 horses and 2 dogs. On the other hand, during an 8-hour

period, no rise in temperature was observed to occur in groups of mice, rats, guinea pigs, or chicks injected intravenously with 10 μ g of this product. Three 20-25 lb chimpanzees were given a single intravenous injection of 1 μ g of lipopolysaccharide. A mean elevation of 2.7°F ensued and the leucocyte count fell to approximately one-half the normal values shortly after injection, followed by a 3-4 fold increase in the number of these cells during a period of 24 hours. The pyrogenic response of man to intravenous injection of the purified endotoxin has not yet been determined.[†] However, in a recent study of its antigenicity in man (13), it was observed that a subcutaneous injection of 20 μ g frequently, but not invariably, resulted in erythema, induration, fever, and malaise, while edema and lymphadenitis were encountered occasionally. On the whole, the reactions exhibited by the purified endotoxin were somewhat more marked than those induced by the conventional typhoid vaccine.* These more pronounced reactions might be expected since the quantity of purified endotoxin administered was greater than that calculated to be contained in the standard dose of 0.5 ml of typhoid vaccine (7.5 μ g).

Activity in Eliciting the Shwartzman Reaction. Local Reaction: A quantitative study was made of the activity of purified endotoxin as a preparing and provoking agent for the phenomenon of local skin reactivity in the rabbit. Groups of animals were prepared for this reaction with amounts of endotoxin varying from 100 to 0.5 μ g. Dilutions of 0.2 ml of this product were injected intradermally at 4 sites on the clean-shaven abdominal skin of each animal. Rabbits, so prepared, were divided into groups of 5-12 animals, and 20 hours later each group was provoked with an intravenous injection of lipopolysaccharide ranging from 100 to 0.2 μ g. The ability of individual animals of the same sex, size and

TABLE IV. Quantitative Assessment of Activity of Typhosa Endotoxin in Production of Local Shwartzman Reaction.

Provocative dose (μ g) admin. i.v. 20 hr after preparatory dose	Preparatory dose (μ g, inj. intraderm.)					
	100	25	10	5	1	.5
100		80*	60	40	40	
25	83	100	57	57	44	10
20	67	50	50		29	
15	67	33	0		0	
10	40	14	14	0	0	
5	40	40	40		20	
1	60				10	10
.2	10				0	0

* Figures indicate % of rabbits exhibiting positive reaction sites as indicated by visible hemorrhage and purpura. Groups of 5-12 New Zealand white rabbits (3 kg) were used.

strain to respond to this component with development of the characteristic local reaction varied; however, in those rabbits capable of responding the incidence of reactivity followed a decreasing gradient as either the preparatory or provocative dose was reduced (Table IV). As would be expected, the extent and severity of the lesions tended to be minimal when the area had been prepared or provoked by the smallest quantities of endotoxin. Nonetheless, it is apparent that as little as 1 μ g, or even less, was sufficient to prepare and provoke this reaction.

The generalized Shwartzman reaction was induced with purified endotoxin from *S. typhosa* in young (2-3 lb) New Zealand white rabbits. Gross and histopathologic examination[†] of the characteristic lesion, bilateral renal cortical necrosis, showed that the pathology evoked by this purified component was in all respects similar to the classical changes induced by crude bacterial products (e.g. occlusion of small blood vessels of kidney with an amorphous fibrinoid-like material. The resultant punctate hemorrhagic and necrotic areas of the cortex occasionally extended into the medullary area) as described by Thomas and Good (7,8). The results of tests in which it was attempted to determine quantitatively

* Adsorption of the lipopolysaccharide on aluminum hydroxide resulted in complete suppression of these toxic manifestations. However, groups of 60 subjects each, inoculated with 20 μ g of adsorbed endotoxin showed relatively poor antibody response (mean O agglutinin titer 1:26) in contrast to those of individuals receiving endotoxin as a saline solution (mean O agglutinin titer 1:90).

[†] The authors are indebted to Lt. Col. Helmuth Sprinz, Walter Reed Army Hospital, who prepared and examined sections of kidneys obtained from rabbits in this study.

TABLE V. Activity of Typhosa Endotoxin in Production of Generalized Shwartzman Reaction.

Preparatory dose of endotoxin (μ g)	Hr elapsed between doses	Provocative dose of endotoxin (μ g)	No. of animals exhibiting bilateral renal cortical necrosis of total inj.*
20	18	50	0/6
20	12	50	4/6
20	8	50	1/2
20	6	50	1/2
10	12	50	1/3
5	12	50	0/4
20	12	100	2/2
20	12	20	3/5
10	12	20	3/4†
10	12	10	2/4†
5	12	10	0/4

* Reaction evident in the gross 18 hr after provocation.

† Degree of reaction was considerably less in these animals.

the amount of endotoxin necessary to prepare and provoke this pathology in the rabbit are given in Table V. The principal variables in these experiments were preparatory dose, provocative dose, and time interval between the 2 injections. The data obtained indicated that production of this reaction does not follow a clear-cut dose-response relationship, but appears to be associated primarily with quantities of endotoxin not far removed from dosage shown to be lethal for the rabbit. The optimal time interval between the two injections, required for production of the characteristic kidney lesion, appeared to be somewhat less than that previously reported by Thomas and Good(7), who employed meningococcal washings as endotoxin.

Discussion. General toxic manifestations associated with administration of typhoid bacilli have been reproduced by a protein-free lipopolysaccharide isolated from an O variant strain of *S. typhosa*. For the most part, these studies have been conducted in the rabbit, a species highly susceptible to endotoxin. However, various animal species differ in their response to the endotoxic action of this product. Thus, man, horse, dog, and rabbit reacted strongly to the pyrogenic and antigenic stimulus of this product. On the other hand, it may be significant that species such as the mouse, rat, guinea pig, and chick, which did not exhibit a thermal response to injection

of 10 μ g of lipopolysaccharide, were the species previously shown to be refractory to this product as an antigen(11). Examination of sera from the latter group by the highly sensitive bacterial agglutination test showed no evidence of production of O antibody following this injection. However, it is not known (and it is difficult to obtain acceptable experimental evidence) whether a causal relationship exists between these two properties. In this connection, it is noteworthy that alterations in the lipopolysaccharide which resulted in decreased antigenic activity were accompanied by reduction in pyrogenicity(11). This lipopolysaccharide possessed antigenicity of a high order in the rabbit, *e.g.* a single intravenous injection of 0.001 μ g elicited production of significant levels of agglutinins(11). The present study shows that in terms of one of the characteristic properties of endotoxin, namely pyrogenic action, this product exhibits extraordinary potency. It is difficult directly to compare and evaluate the pyrogenic potency of this product with that of the various fever-producing agents reported in the literature; nonetheless, the finding that the minimum pyrogenic dose for a 3 kg rabbit is approximately 0.0002 μ g affords evidence that this typhosa lipopolysaccharide is one of the most potent pyrogens known. It has been shown that these attributes of pyrogenicity and antigenicity in the rabbit are manifested in essentially the same range of concentrations and exhibit similar extinction values(11). Depending on the species involved, lipopolysaccharide evokes either both effects, or neither. These findings may therefore be indicative of a common functional mechanism. Experimental evidence tending to support the thesis that these physiological activities of endotoxin may be responsible for marked antigenic activity, subsequently expressed by the host, was obtained in our studies on enhancement of antibody response to proteins by typhosa lipopolysaccharide(14).

The remarkable activity of this purified bacterial component in eliciting these experimental host reactions may provide further insight into the classical reactions encountered clinically in those diseases of man caused by

endotoxin-bearing organisms. For example, calculations based on rhamnose content and on actual isolation, indicated that 1 billion organisms (*S. typhosa*, 0-901) can contribute approximately 15 μg of lipopolysaccharide. That the minimum pyrogenic dose is approximately 0.0002 $\mu\text{g}/3$ kg rabbit, makes it apparent that endotoxic material released from relatively few bacteria (1×10^4) can produce fever in the rabbit. Man is considered to be more susceptible to endotoxin than the rabbit (15), and this study offers further evidence for the hypothesis advanced by Favorite and Morgan that under conditions of natural infection, extremely small quantities of endotoxin may elicit the pyrexial response in the typhoid fever patient.

A similar situation may apply in the Schwartzman reaction. It is apparent that rabbits vary in ability to react to the endotoxin used by us. The dose-response relationship of an individual rabbit is such that if susceptible to this reaction, it will respond over a wide range of concentrations of endotoxin. That nonsusceptible rabbits exhibit no reduction in their refractory state even when doses of endotoxin are greatly increased further attests to the "all or none" nature of this phenomenon. A comparable individual hyperreactive state has been postulated by Black-Schaffer *et al.* as being a prerequisite for the occurrence in man of the pathology characteristic of the Schwartzman reaction (16).

Summary. A study was made of the endotoxic properties of a protein-free lipopolysaccharide isolated from the 0-901 strain of *S. typhosa*. The product was lethal for rabbits in a dose range of 20-50 μg while 300-500 μg were required to kill mice. The minimum pyrogenic dose in rabbits was 0.0002 μg , characterizing it as a pyrogen of extremely high potency. In a quantitative study of the local Schwartzman reaction, 1 μg or less, was effective as a preparatory or provocative dose. Its activity in eliciting bilateral renal cortical

necrosis, the lesion characteristic of the generalized Schwartzman reaction, was in the range of 5-20 μg . Thus, very small quantities of a lipopolysaccharide, freed of protein, are capable of producing the various biological alterations generally attributed to endotoxins.

Addendum. Since this report was submitted for publication preliminary tests of the effects of the typhosa lipopolysaccharide in man were undertaken (17). These indicate that when injected i.v. the minimum pyrogenic dose for a 70 kg subject lies in the range of .01-.04 μg .

1. Burrows, W., *Ann. Rev. Microbiol.*, 1951, v5, 181.
2. Bennett, I. L., and Beeson, P. B., *Medicine*, 1950, v29, 365.
3. Thomas, L., *Ann. Rev. Phys.*, 1954, v16, 467.
4. Schwartzman, G., *Phenomenon of Local Tissue Reactivity*, Paul B. Hoeber, Inc., New York, N. Y., 1937.
5. Morgan, H. R., *Am. J. Path.*, 1943, v19, 135.
6. Delaunay, A., Boquet, P., Lebrun, J., Lehoul, Y., and Delaunay, M., *J. Phys. (Paris)*, 1948, v40, 89.
7. Thomas, L., and Good, R. A., *J. Exp. Med.*, 1952, v96, 605.
8. Good, R. A., and Thomas, L., *ibid.*, 1952, v96, 625.
9. Creech, H. J., and Hankwitz, Jr., R. F., *Cancer Research*, 1954, v14, 824.
10. Webster, M. E., Sagin, J. F., Landy, M., and Johnson, A. G., *J. Immunol.*, 1955, v74, 455.
11. Landy, M., Johnson, A. G., Webster, M. E., and Sagin, J. F., *ibid.*, 1955, v74, 466.
12. Staub, A. M., and Combes, R., *Ann. Inst. Pasteur*, 1951, v80, 21; 1952, v83, 528.
13. Landy, M., Gaines, S., Seal, J. R., and White-side, J. E., *Am. J. Pub. Health*, 1954, v44, 1572.
14. Johnson, A. G., Gaines, S., and Landy, M., *Fed. Proc.*, 1954, v13, 499.
15. Favorite, G. O., and Morgan, H. R., *J. Clin. Invest.*, 1942, v21, 589.
16. Black-Schaffer, B., Hiebert, T. G., and Kerby, G. P., *Arch. Path.*, 1947, v43, 28.
17. Sanford, J. P., to be published.

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Some Effects of ω -Methyl Analogues of Vitamin B₆ in Rats. (21938)

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ω -Methylpyridoxine (2-ethyl-3-hydroxy-4,5-bis(hydroxymethyl)pyridine), in which the methyl group of pyridoxine has been replaced by an ethyl group(1), was one of the first analogues of vit. B₆ to be synthesized and tested in living organisms. Robbins found the compound replaced pyridoxine in promoting growth of excised tomato roots(2), but was toxic for *Ceratostomella ulmi*(3). Corresponding analogues of pyridoxal and pyridoxamine also have been synthesized(4). For lactic acid bacteria such as *Streptococcus faecalis*, these latter compounds replace pyridoxal as growth factors under some conditions(4-6), but may inhibit growth under slightly different conditions, depending upon the vit. B₆-dependent process which limits growth(5,6). Like ω -methylpyridoxine(7), they inhibit growth of yeast (*Saccharomyces carlsbergensis*); this inhibitory effect is overcome completely by pyridoxine, pyridoxamine, or pyridoxal.

A single dose of ω -methylpyridoxine was less than 2% as effective as pyridoxine in alleviating symptoms of vit. B₆ deficiency in rats(1). No other reports of the physiological effects of these compounds in animals have appeared; results of such an investigation are presented below.

Materials and methods. Synthesis of ω -methylpyridoxal and ω -methylpyridoxamine has been described earlier(4). ω -Methylpyridoxine(1) and an authentic sample of the lactone of 4-pyridoxic acid(8) were gifts from Dr. Karl Folkers. Weanling albino rats were caged individually, and fed vit. B₆-deficient ration of Sarma *et al.*(9) modified by substituting vitamin-free casein (Labco) for blood fibrin. Supplements were added to the diet or given separately by stomach tube. In the latter event a tuberculin syringe and a dulled no. 15 needle, bent and cut off at the proper length, were used. Food and water were given *ad libitum*.

Results. a. Activity of ω -methylanalogues

of vit. B₆ during preventive assay. Weanling rats (Sprague-Dawley) were fed the deficient diet supplemented with pyridoxal, one of the analogues, or both pyridoxal and analogue, as indicated in Table I. Over a 4-week period, the ω -methyl analogues possess considerable growth-promoting activity. However, the higher levels of analogues were no more effective than the lower levels, and no amount of analogue permitted a maximum growth rate. The compounds were not irreversibly toxic, for when fed together with pyridoxal, a high rate of growth was achieved.

When supplementation was continued for a longer time, it became evident that the high level of all 3 ω -methyl analogues permitted even less growth than the lower level. Such results are shown for ω -methylpyridoxal in Fig. 1. Eventually the rate of growth of supplemented animals slowed to that of the deficient controls. Thus, while the analogues permit growth for a limited length of time in animals containing initially adequate supplies of vit. B₆, they become unable to support growth once bodily supplies of vit. B₆ reach a sufficiently low level.

More striking evidence than growth rates, of the inability of these analogues fully to replace vit. B₆ was provided by the observed symptomatology. Beginning about 2 weeks after supplementation with the analogues, practically all animals of Groups 5 through 10, Table I, underwent frequent epileptiform fits, especially during weighing periods. Occurrence of such convulsions has been noted frequently in animals fed vit. B₆-deficient diets alone (*e.g.* 10), but is not a prominent feature of vit. B₆ deficiency on this basal ration, and was not noted in any of the deficient animals of Group 1 (Table I) until fully 4 weeks after their occurrence had become common in the analogue-fed animals. Even then, the incidence of the attacks in deficient Group 1 was not more than 25% of that in analogue-fed animals. The 3 analogues, therefore, pro-

TABLE I. Comparative Effects of Pyridoxal and ω -Methyl Analogues of Vitamin B₆ on Growth of Rats. Each group contained 6 animals.

Group	Supplement	Amt of supplement/day, γ	Wt gain in 4 wk, g	Apparent molar activity (pyridoxal=100)
1	0	—	46	
2	Pyridoxal	1.6	71	
3	"	4.1	94	
4	"	8.2	114	
5	ω -Methylpyridoxal	16.6	100	32
6	"	83	100	6.5
7	ω -Methylpyridoxamine	17.8	92	23
8	"	88	101	6.4
9	ω -Methylpyridoxine	16.6	100	32
10	"	83	93	5.1
11	Pyridoxal + ω -methylpyridoxal	82	140	
12	Pyridoxal + ω -methylpyridoxamine	88	134	
13	Pyridoxal + ω -methylpyridoxine	82	126	
		83		

duce a deficiency of a type in which these fits are an unusually common feature—an observation of interest in view of the observed occurrence of convulsions in infants receiving suboptimal amounts of vit. B₆(11). No such attacks occurred in animals of groups 11-13, which received pyridoxal together with the high level of analogue.

b. Activity of ω -methyl analogues of vit.

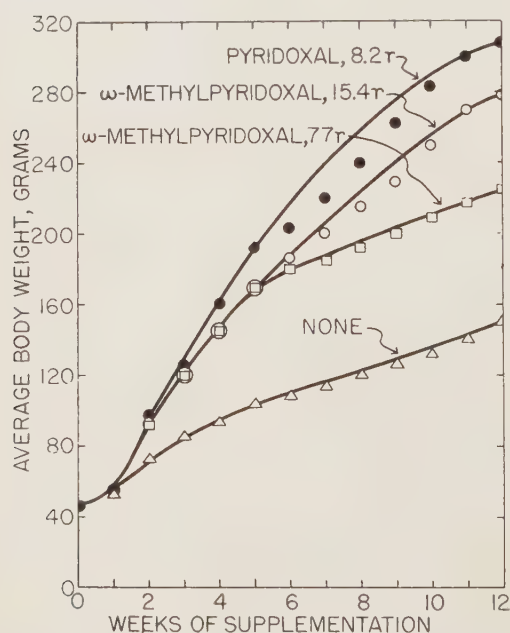


FIG. 1. Effect of time on comparative growth-promoting activities of pyridoxal and ω -methylpyridoxal for rats. Supplements indicated were administered daily.

B₆ during curative assay. The apparent ability of the analogues to support growth only during the first few weeks (Fig. 1) suggests that as depletion of vit. B₆ proceeds, growth responses to the analogue would become less marked. This was checked by holding weanling animals (derived from the Wistar strain) on the deficient ration for 5 weeks, then supplementing the diet with high levels of ω -methylpyridoxine. A pronounced stimulation of growth occurred in the analogue-fed group during the first week, which decreased greatly in the course of the second and third weeks to a value below that of the deficient control group (Table II). Four of the 5 animals of the analogue-supplemented group died between the 34th and 41st days of supplementation, and the experiment discontinued. None of the deficient control animals had died during this period. This high level of ω -methylpyridoxine was non-toxic when fed together with pyridoxine. Thus the unusual result is observed that a compound that apparently corrects early deficiency symptoms eventually makes these symptoms more pronounced, and changes the intensity of individual symptoms relative to one another.

c. Metabolism of ω -Methylpyridoxine in Rats. Application of the method of Heyl(8) for synthesis of 4-pyridoxic acid to a small sample of ω -methylpyridoxine yielded a product with an ultra-violet absorption spectrum essentially identical with that of 4-pyridoxic

TABLE II. Effect of ω -Methylpyridoxine on Growth and Survival of Vit. B₆ Deficient Rats. Each group contained 5 animals.

Supplement	Amt fed per day, γ	Avg wt gain/wk during					Survivors at 41 days
		1st	2nd	3rd	4th	5th wk	
0		3.2	1.3	.9	3.3	.5	5
Pyridoxine	8.2	27	16	15	16	8.3	5
ω -Methylpyridoxine	820	28	7.9	1.0	.1	-10.5	1

acid. After treatment in such a way as to form the lactone(12) the absorption spectrum proved identical with that of 4-pyridoxic acid lactone. On paper chromatograms the product migrated somewhat faster than 4-pyridoxic acid in all solvents, as might be expected from its slightly less polar character. The method of synthesis, the spectrum of the free acid, and the spectrum of the lactone, all demonstrate that the product is ω -methyl-4-pyridoxic acid. Different animals excrete variable proportions of administered pyridoxine as 4-pyridoxic acid(13); pyridoxal and pyridoxamine are also oxidized to this same product(14). To determine whether ω -methylpyridoxine was similarly metabolized, rats were fed high levels of ω -methylpyridoxine (4600 $m\mu$ moles/day) of pyridoxine (4900 $m\mu$ moles/day) for 24 hours. Chromatographic comparisons (Table III) showed that administration of ω -methylpyridoxine gave rise to ω -methylpyridoxic acid in the urine. Quantitative determination by the method of Møller(12) showed, in agreement with results of Huff and Perlzweig(13), that only about 0.5% of administered pyridoxine was recovered as 4-pyridoxic acid; similarly, 0.6% of the administered ω -methylpyridoxine was re-

covered in the urine as ω -methyl-4-pyridoxic acid. In neither case are the other products of metabolism known.

d. *Some Effects of ω -Methylpyridoxine Administration on Enzyme Levels in Tissues.* D-Amino acid oxidase, the glutamic-aspartic transaminase, and thionase (cysteine desulphydrase) were selected as representative of one vit. B₆-independent and 2 vit. B₆-dependent enzymes and their levels in appropriate tissues from normal (Group 13, Table I), deficient (Group 1, Table I), and analogue-fed (Group 10, Table I) animals compared. Animals were killed by decapitation, the tissues excised and homogenized. Results (Table IV) show no diminution in activity of the vit. B₆-independent kidney D-amino acid oxidase, but demonstrate a moderate reduction in activity of liver transaminase and a severe reduction in liver thionase in both vit. B₆-deficient and analogue-fed animals. The differences in symptomatology observable in simple vit. B₆-deficiency and in analogue-fed animals thus cannot be explained in terms of the effects on these 2 enzymes, but must result from differential effects upon one or more of the multitude of other vit. B₆-dependent enzymes of tissues. Rates of diminution in enzyme activity, an effect not considered in the above comparisons, probably are also of importance here.

The result shows that the ω -methyl analogues are not the functional equivalent of vit. B₆ in activation of these enzymes, and if they are converted to ω -methylpyridoxal phosphate by animal tissues (as they are by bacteria (5,6)) the latter must be *comparatively* ineffective as an activator of these two enzymes. This was checked by comparing the effectiveness of ω -methylpyridoxal phosphate(4) and pyridoxal phosphate as activators of enzyme preparations obtained from rats fed the vit. B₆-deficient ration for 37 days. The analogue coenzyme activated liver glutamic-aspartic

TABLE III. Excretion of ω -Methyl-4-Pyridoxic Acid by Rats Fed ω -Methylpyridoxine. Approximately 30 $m\mu$ moles (.01 ml) of acids or lactones and 0.05 ml of urine samples were applied to 18 $\frac{1}{4}$ by 22 $\frac{1}{2}$ inch sheets of Whatman No. 1 filter paper, and developed with butanol-ethanol-water (4:1:1) in an ammoniacal atmosphere. Zones of these fluorescent substances were detected by observation under ultra-violet light.

Substance chromatographed	R _f values	
	Un-treated	Treated for lactone formation
4-Pyridoxic acid	.68	.40
ω -Methyl-4-pyridoxic acid	.77	.53
Urine of pyridoxine-fed rats	.66	.44
Urine of ω -methylpyridoxine-fed rats	.77	.46

TABLE IV. Comparison of Kidney D-Amino Acid Oxidase, Heart Glutamic-Aspartic Transaminase and Hepatic Thionase between Normal, Vitamin B₆-Deficient, and ω -Methylpyridoxine-Fed Rats. Each determination was carried out on pooled tissues of 3 animals. Normal and analogue-fed rats received vit. B₆-free diet and supplements for 86 days from weaning. Vit. B₆-deficient rats received the vit. B₆-free diet only for 49 days.

Treatment of animal	D-Amino acid oxidase,* μ l O ₂ /hr/mg wet wt	Enzyme systems —	
		Transaminase,† μ l CO ₂ /10 min./mg wet wt	Thionase,‡ μ g H ₂ S/2 hr/g wet wt
Normal	1.4	6.2	166
Analogue-fed	1.8	4.5	45
Vit. B ₆ -deficient	1.3	4.4	25

* By method of Armstrong, *et al.* (15).

† " " " Ames and Elvehjem (16).

‡ " " " Thompson and Guerrant (17).

transaminase, but was only 11% as active on the weight basis as pyridoxal phosphate. Liver thionase was not fully reactivated by any concentration of the analogue coenzyme; the partial reactivation achieved required over 50 times the concentration that sufficed when pyridoxal phosphate was the coenzyme. The behavior of these enzymes of mammalian origin to the analogue coenzyme resembles closely that of the corresponding bacterial enzymes (6).

Discussion. Under appropriate conditions in certain bacteria, ω -methylpyridoxal and ω -methylpyridoxamine support continued growth because they are converted to ω -methylpyridoxal phosphate, which activates the essential vit. B₆-dependent enzyme in place of pyridoxal phosphate (5,6). Under closely related conditions, when another set of vit. B₆-dependent enzymes is made limiting, the same vitamin analogues inhibit growth, presumably because these vit. B₆-dependent enzymes are not activated by the analogue coenzyme, which consequently behaves as a structural antagonist. That similar relationships hold in animal systems seems probable from the observations that ω -methylpyridoxine and pyridoxine are metabolized similarly, and that ω -methylpyridoxal phosphate varies in its ability to activate various vit. B₆-dependent enzymes of the rat. The observed facts that these analogues first promote, then inhibit, growth on vit. B₆-free rations might be readily interpreted in terms of

these effects. It may be supposed that as the stores of vit. B₆ within the body become depleted, various vit. B₆-dependent enzymes become hypofunctional in turn, depending, upon their affinities for pyridoxal phosphate (*cf.* 18). If the first of these to become hypofunctional were also activated by ω -methylpyridoxal phosphate, this analogue would be expected to promote growth. When, however, additional essential vit. B₆-dependent enzymes that are not activated by the analogue coenzyme become hypofunctional, then the analogues no longer promote growth. Since the analogues act also as displacing agents, pyridoxal phosphate displaced by the analogue coenzyme from one apoenzyme may become available for activation of a second apoenzyme having different relative affinities for the coenzyme and analogue coenzyme.* This displaced pyridoxal phosphate may initially contribute to the growth effects of the analogue. At the same time, however, if the enzyme from which this pyridoxal phosphate has been displaced itself catalyzes reactions essential for growth, and is not activated (or is activated to an insufficient extent) by available concentrations of the analogue coenzyme, such displacement would explain the active toxicity exhibited by the analogues after more prolonged periods of administration. Since the enzymes from which pyridoxal phosphate is displaced are not necessarily the same as those that would lose this coenzyme first in simple vitamin deficiency,† administration of the analogue might be expected to produce a pattern of deficiency symptoms differing in some respects from those produced by simple deficiency, as is actually observed. Finally, a part of the vit. B₆ ingested by the animal normally is made unavailable for growth processes through oxidation to 4-pyridoxic acid (and unidentified products) and excretion. The participation of ω -methylpyridoxine as a competitive substrate in these latter reactions would decrease the amount of vit. B₆ destroyed in this way, and thus spare additional amounts for essential metabolic uses.

The similarity of these observations to

* That different enzymes of a single organism may show such varying affinities has been demonstrated in *Streptococcus faecalis* (5,6).

those made with "diethylriboflavin"(20) and with lyxoflavin(21) suggests a fairly widespread occurrence of similar phenomena.

Summary. ω -Methylpyridoxal, ω -methylpyridoxamine and ω -methylpyridoxine support growth of rats for several weeks following deletion of vit. B₆ from the ration. Eventually, however, growth rate in the presence of these compounds slows, and becomes equal to or less than that of vit. B₆-deficient control animals. The period of growth promotion is decreased greatly when the compounds are administered to depleted rats. Following this period of growth-promotion, growth with high concentrations of ω -methylpyridoxine is distinctly less than that in control animals; and death results well before any deaths are noted in the control group. In uncomplicated vit. B₆ deficiency on this ration convulsive seizures are infrequent and were observed only following approximately 6 weeks on the deficient diet. In contrast, in the animals fed the ω -methyl analogues of vit. B₆ convulsions occurred frequently, in all animals, starting about 2 weeks after supplementation while substantial weight gains were still occurring. All of these symptoms were prevented by feeding pyridoxal together with the analogues. Following administration of ω -methylpyridoxine to animals, ω -methyl-4-pyridoxic acid appears in the urine in amounts similar to those found for 4-pyridoxic acid following pyridoxine administration. Glutamic-aspartic transaminase and thionase are reduced in ω -methylpyridoxine-fed animals to about the levels found during uncomplicated vit. B₆ deficiency. Correspondingly, ω -methylpyridoxal phosphate was only about 11% as active as pyridoxal phosphate in activating the glutamic-aspartic transaminase, and did not fully

activate liver thionase at any concentration. The facts that feeding these analogues first promotes, but eventually inhibits growth, and produces a deficiency of a different type than that found in uncomplicated vit. B₆-deficiency, may be interpreted in terms of (a) the dual character of ω -methylpyridoxal phosphate as an activator for some vit. B₆-dependent enzymes, but not for others, (b) its role as a structural antagonist for vit. B₆ in those enzymes that it does not activate, and (c) the role of these analogues as competitive substrates for vit. B₆ in reactions leading to destruction of the vitamin.

1. Harris, S. A., and Wilson, A. N., *J. Am. Chem. Soc.*, 1941, v63, 2526.
2. Robbins, W. J., *Am. J. Bot.*, 1942, v29, 241.
3. Robbins, W. J., and Ma, R., *Bull. Torrey Bot. Club*, 1942, v69, 342.
4. Ikawa, M., and Snell, E. E., *J. Am. Chem. Soc.*, 1954, v76, 637.
5. Olivard, J., and Snell, E. E., *J. Biol. Chem.*, 1955, v213, 203.
6. ———, *ibid.*, 1955, v213, 203.
7. Rabinowitz, J. C., and Snell, E. E., *Arch. Biochem. and Biophys.*, 1952, v43, 408.
8. Heyl, D., *J. Am. Chem. Soc.*, 1948, v70, 3434.
9. Sarma, P. S., Snell, E. E., and Elvehjem, C. A., *J. Biol. Chem.*, 1946, v165, 55.
10. Chick, H., El Sadr, M. M., and Worden, A. V., *Biochem. J.*, 1940, v34, 595.
11. Maloney, C. J., and Parmelee, A. H., *J. Am. Med. Assn.*, 1954, v154, 405.
12. Møller, P., *Acta Chem. Scand.*, 1951, v5, 1418.
13. Huff, J. W., and Perlzweig, W. A., *J. Biol. Chem.*, 1944, v155, 345.
14. Rabinowitz, J. C., and Snell, E. E., *PROC. SOC. EXP. BIOL. AND MED.*, 1949, v70, 235.
15. Armstrong, K. F., Feldott, G., and Lardy, H. A., *ibid.*, 1950, v73, 159.
16. Ames, S. R., and Elvehjem, C. A., *J. Biol. Chem.*, 1946, v166, 81.
17. Thompson, R. Q., and Guerrant, N. B., *J. Nutrition*, 1953, v50, 161.
18. Snell, E. E., *Physiol. Rev.*, 1953, v33, 509.
19. Dietrich, L. S., and Shapiro, D. M., *PROC. SOC. EXP. BIOL. AND MED.*, 1953, v84, 555.
20. Lambooy, J. P., and Aposhian, H. V., *J. Nutrition*, 1952, v47, 539.
21. Snell, E. E., Klatt, O. A., Bruins, H. W., and Cravens, W. W., *PROC. SOC. EXP. BIOL. AND MED.*, 1953, v82, 583.

† Dietrich and Shapiro(19) have observed that the enzymatic disabilities produced by feeding 4-desoxy-pyridoxine to rats do not coincide exactly with those produced by simple vitamin deficiency. This result should be expected, since the degree to which an analogue displaces pyridoxal phosphate depends not only upon the dissociation constant of the various proteins for pyridoxal phosphate, but also upon their affinity for the analogue coenzyme and upon the rate at which the enzyme is inactivated and reformed within the tissues.

Changes in Serum Proteins Produced by Infusions of Dextran. (21939)

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(Introduced by David Seligson.)

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In the course of studies of possible metabolic effects of dextran infusions, a patient with chronic liver disease was given repeated infusions of dextran. A marked decrease in the concentration of serum proteins was observed, with a greater reduction in globulin than in albumin. Detailed studies were undertaken to confirm and evaluate these particular findings. For this purpose, 4 additional patients with liver disease and 8 patients convalescent from diseases other than liver disease were studied.

Methods. One patient was given only a single infusion of dextran, 750 cc of 12% dextran in water, the other 12 received daily infusions of 1000 cc of 6% dextran in saline. Five of these 12 men were given infusions for 4 successive days, 4 for 5 days, one for 6 days, and 2 for 15 days. Other than prolonged bleeding time(1), slightly prolonged one-stage prothrombin time, and minor headaches, no difficulties were encountered. Some patients gained as much as 3 kg of weight, but none developed overt edema.

Serum proteins were measured daily in most cases by the method of Reinhold(2), in which the globulins are precipitated by a sulfate-sulfite solution, and the protein in solution measured by the biuret reaction. On selected serums drawn both before and after dextran infusions the serum proteins were also checked by sulfate-sulfite precipitation and the analysis of supernatant nitrogen content by the Kjeldahl method. Filter paper electrophoresis was also used to check the partition of serum proteins, using the dye elution technique for colorimetric quantitation of the bromphenol blue bound by each protein zone. Dextran levels were measured by the turbidity method(3). In 3 of the liver disease patients serum cholinesterase(4), zinc turbidity(5), and thymol turbidity(6) were also measured.

Results. With repeated infusions of dex-

tran, levels of dextran as high as 2.5 g % were found in the serum. Dextran was but slowly removed; appreciable levels were still present in the serum 10 to 15 days after infusion. Hematocrits and total serum protein concentrations changed inversely with the dextran level. In general the sum of the concentrations of protein and dextran remained constant, and roughly equalled the total pro-

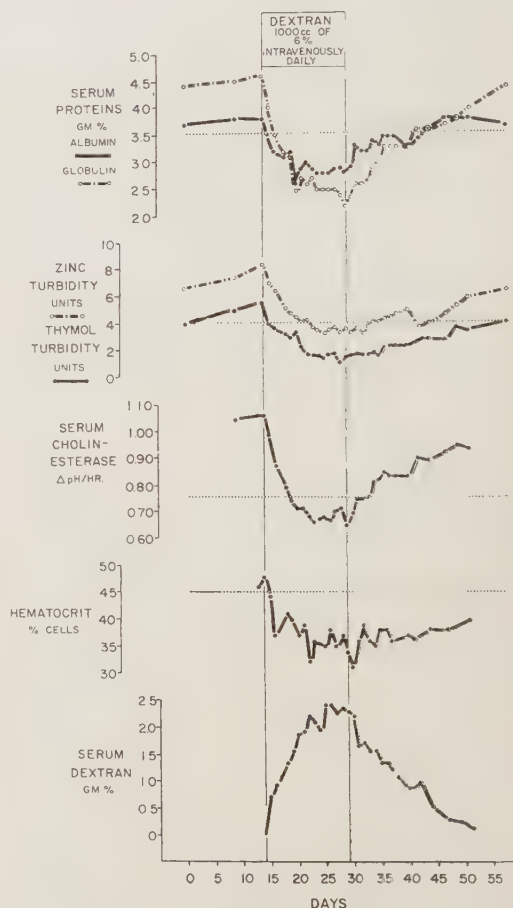


FIG. 1. Changes in serum proteins, hematocrit, serum dextran, serum cholinesterase, and zinc and thymol turbidity tests with repeated infusions of dextran in a patient with biliary cirrhosis convalescent from surgical relief of jaundice.

TABLE I. Correlations among Serum Proteins, Serum Dextran, and Hematocrit.

	Dextran, g %	Change in hematocrit, % cells
Change in total protein, g %	-.80	+.61*
" " albumin, " "	-.77	+.44
" " globulin, " "	-.76	+.63*
" " A/G ratio	+.57	-.55
" " hematocrit, % cells	-.44	—

All correlations are highly significant. With 80 degrees of freedom (all correlations are based on 83 or more pairs) a correlation coefficient of .28 has a probability of .01.

* Correlation coefficients between percentage changes in serum proteins and hematocrit differ from those above by .03 or less.

tein concentration before dextran infusion.

In all patients, both liver disease patients and others, the serum globulin concentration decreased more than that of the serum albumin, so that the albumin:globulin ratios rose. These differential changes in albumin and globulin fractions were observed by both chemical methods used for analysis, and also in the paper electrophoresis patterns. By the latter technique the various globulins were found to be reduced equally.

In the patients in whom it was measured, the serum cholinesterase fell with dextran infusion. Abnormally high zinc and thymol turbidity values fell to the low normal range. (Fig. 1).

Discussion. Part of the reduction of serum protein concentration was certainly caused by expansion of the plasma volume by dextran. However, the increase in plasma volume calculated from changes in hematocrit accounted for only a part of the gain in body weight. Also it may be seen in Table I that the protein changes were less well correlated with the hematocrit changes than with the serum concentrations of dextran. The different percentage changes in albumin and globulin cannot be explained by plasma volume expansion. This difference was not caused by interference by dextran with the analytic method, because similar changes were found by the paper electrophoresis technique. Similar changes have been observed by Jaenike and Waterhouse (7).

Expansion of the interstitial fluid as well

as the plasma volume must have occurred in these patients. Since the molecular size of the globulins is much greater than that of albumin the rate of transfer of globulins across the capillary wall should be slower than that of albumin. Therefore with transfer of fluid and protein from the plasma to interstitial fluid to compensate for the hypervolemia, the albumin concentration should fall faster than that of the globulin. A significant fall in total circulating albumin, but not in globulin, has been observed shortly after a rapid infusion of dextran (8). Later as dextran is metabolized and as fluid and dextran are excreted, albumin should return more rapidly to the plasma than globulin. This process of reequilibration may possibly explain the relatively greater reduction of globulin than of albumin which was observed.

The changes in albumin and globulin concentrations may also be explained by reductions in rates of synthesis. Since serum cholinesterase activity seems to parallel the rate of albumin synthesis (9), the observed reduction in cholinesterase may be taken as evidence of a decreased rate of albumin synthesis. Colloids similar to dextran are taken up by the reticulo-endothelial system, the site of globulin synthesis. Thus dextran might reasonably be expected to depress globulin synthesis. If dextran does reduce the rates of synthesis of albumin and globulin, there is no reason to expect, *a priori*, that the reductions need be the same.

The changes to normal of elevated zinc and thymol turbidity values are doubtless secondary to the relative changes in albumin and globulin.

Summary. Daily infusions of dextran were given to 13 patients for as long as 15 days. In addition to the decrease in total serum protein from plasma volume expansion, dextran was found to produce a relatively greater decrease in globulin than in albumin. Serum cholinesterase activity decreased, and zinc and thymol turbidity values fell.

1. Carbone, J. V., Furth, F. W., Scott, R., Jr., and Crosby, W. H., *Proc. Soc. Exp. Biol. and Med.*, 1954, v85, 101.

2. Reinhold, J., *Standard Methods of Clinical*

Chemistry, Academic Press, Inc., New York, 1953, v1, 88.

3. Metcalf, W., and Rousselot, L. M., *J. Lab. Clin. Med.*, 1952, v40, 901.

4. Michel, H. O., *ibid.*, 1949, v34, 1564.

5. Kunkel, H. G., *Proc. Soc. Exp. Biol. and Med.*, 1947, v66, 217.

6. Shank, R. E., and Hoagland, C. L., *J. Biol. Chem.*, 1946, v162, 133.

7. Jaenike, J. R., and Waterhouse, C., *Circulation*, 1955, v11, 1.

8. Waldstein, S. S., Carbone, J. V., and Furth, F., to be published.

9. Vorhaus, L. J., Scudamore, H. H., and Kark, R. M., *Gastroenterology*, 1950, v15, 304.

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Effect of High Levels of Fat, Lactose, and Type of Bulk in Guinea Pig Diets.* (21940)

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Woolley and Sprince(1) reported that supplements of certain inert materials, such as powdered cellophane and Cellu flour, improved the growth of guinea pigs fed purified diets. Booth *et al.*(2) who found a similar beneficial action of bulk, reported gum acacia (formerly called gum arabic) to be slightly superior to Cellu flour. However, Reid and Briggs(3) could not always obtain consistent growth with diets containing gum acacia, and turned to the use of Spangles,[†] a coarsely-ground cellophane product.

In this paper we are presenting further studies on the effect of different types of bulk, especially Solka-floc,[‡] a very pure, finely-ground and relatively inexpensive cellulose product, on the growth of guinea pigs as well as the effect of different levels of fat and lactose.

Methods. To test the effectiveness of these different materials, 3 types of diets were devised, including a "normal", a high-fat 24% corn oil, and one with lactose as the only carbohydrate. The latter 2 diets were chosen because more information was desired on the effect of these substances on the deposition of liver fat in guinea pigs fed milk diets(4). The

basal or normal diet used in this experiment provided per 100 g of ration in g: sucrose, 43; casein, 30; bulk, 15; salts IV, 4(5); corn oil, 4; potassium acetate, 2.5; choline-Cl, 0.75; magnesium oxide, 0.50; and vitamin mix, 0.25. The vitamin mix provided per 100 g of ration in mg: inositol, 200; niacinamide, 20; p-aminobenzoic acid, 10; Ca-pantothenate, 8; riboflavin, 3; thiamine-HCl, 2; pyridoxine-HCl, 2; folic acid, 1; in μ g: biotin, 100; B₁₂, 4. Fat soluble vitamins were provided weekly by dropper in the following amounts: A, 2000 I.U.; D, 20 I.U.; E, 12 mg; K, 0.2 mg. It should be noted that the diets contained 2.5 times the level of choline normally used in guinea pig diets to allow for any greater need with high-fat diets. The addition of corn oil to the high-fat diets and to those with graded levels of corn oil was made at the expense of sucrose. The care and feeding of guinea pigs have been described elsewhere(6). Since the diets differed so widely, the guinea pigs were initially divided into 3 groups with each group gradually being adapted to its particular diet. After adaptation, each of the 3 dietary groups was subdivided into 3 groups of 5 guinea pigs each, with the same average starting weight within each main group (normal, 194 g; high-fat, 221 g; and lactose, 217 g). The 3 normal groups were on experiment for 4 weeks after which they were redistributed into groups averaging 380 g. These groups were fed diets with 4, 8,

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[†] Rayon Processing Co., Pawtucket, R. I.

[‡] Brown and Co., Berlin, N. H.

TABLE I. Comparison of Gum Acacia, Spangles and Solka-floc as Bulk for the Guinea Pig.

Diet and time	Avg growth in g/day		
	Gum acacia	Spangles	Solka-floc
Normal, 4 wk	6.5	6.1	7.4
High-fat, 6 wk	3.8	4.5	4.9
(% liver fat wet basis)	(5.1)	(5.4)	(4.3)
Lactose, 2 wk	1.0	1.8	2.0
Lactose, 4 more wk			2.6
1½ lactose & ½ sucrose, 4 wk			5.6
Graded levels of corn oil	4%	8%	12%
(Solka-floc) 3 wk	5.8	5.3	7.7

and 12% levels of corn oil with Solka-floc as the bulk for 3 weeks. The 3 groups receiving high-fat diets were on experiment for 6 weeks, after which the livers were analyzed for fat by the method of Bixby *et al.* (7). The groups receiving lactose as the carbohydrate were on experiment for 2 weeks, after which the 12 remaining animals were redivided into 2 groups. One group continued to receive lactose as the only carbohydrate, and the other group had half of the lactose replaced by sucrose. Both groups received Solka-floc as the bulk, and were on experiment for 4 weeks.

Results and discussion. The results in Table I show that of the 3 types of bulk, Solka-floc supported slightly better growth with each type of diet. The feces from the guinea pigs receiving Solka-floc were the most normal in appearance. For these and other reasons previously mentioned, Solka-floc was used as the bulk in all subsequent guinea pig diets.

The general appearance of the guinea pigs receiving the high-fat diet with gum acacia, was definitely inferior to that of the other groups, probably due in part to the fact that gum acacia is less absorbent and left a more oily ration, which tended to stick to the fur of the animals. The livers of the animals receiving the high-fat diets were almost normal in appearance and fat in content. Although the livers of the group receiving Solka-floc had slightly less fat, none of the livers were very fatty since a normal guinea pig liver will average about 4% fat.

With high-fat diets, one animal died in the group receiving gum acacia and also one in

the group receiving Spangles. However, the relatively good growth observed with the group receiving high-fat with Solka-floc was somewhat surprising. This suggested that the guinea pig could utilize high levels of corn oil. Consequently, the groups of animals that had been receiving the normal diets were redistributed and given diets with graded levels of corn oil. It is apparent from Table I that the group receiving 12% corn oil grew very well, somewhat better than the groups receiving 4 or 8% corn oil. As a result of this finding the corn oil content of all guinea pig diets for subsequent experiments was raised to 7.4% to permit easier mixing and to lessen the wastage of rations that become rather dry and loose with the use of Solka-floc and only 4% corn oil.

The guinea pigs receiving lactose as the sole carbohydrate grew very poorly, as can be seen by the growth of only 2.0 g or less/day. One animal from each group died within the first 2 weeks. When the remaining animals were redivided into 2 groups, the substitution of sucrose for ½ the lactose in this diet resulted in a marked improvement in growth. This group gained an average of 5.6 g/day for 4 weeks as compared to the growth of only 2.6 g/day for the group continuing to receive lactose as the only carbohydrate. This would indicate that there is very little adaptation by the guinea pig to utilize lactose under these conditions.

The results of these experiments would also suggest that the poor growth of guinea pigs fed milk diets is primarily due to the lactose in the diets, although preliminary evidence (4) indicates that these diets may also be deficient in potassium, magnesium, arginine, and possibly other substances. Although these experiments do show that guinea pigs can utilize 12% corn oil very effectively and 24% rather well, they do not shed much light on the cause of the very fatty livers found in guinea pigs fed milk diets. The possibilities still exist that deficiencies in vitamins or amino acids or differences in kind and level of fat may be etiological factors in this condition.

Summary. Gum acacia, Spangles, and Solka-floc have been compared as bulk agents

for the guinea pig by employing normal, high-fat, and lactose diets. The average growth rate of the groups receiving Solka-floc was slightly better in all cases. Guinea pigs grew very well on diets containing 12% corn oil (7.7 g/day) and at a rate of 4.9 g/day with 24% corn oil diets using Solka-floc as the bulk. Guinea pigs fed diets with lactose as the sole carbohydrate grew at a rate of about 2.0 g/day. Substitution of sucrose for half of the lactose increased growth to over twice the rate obtained with lactose alone.

1. Woolley, D. W., and Sprince, H., *J. Biol. Chem.*,

1945, v157, 447.

2. Booth, A. N., Elvehjem, C. A., and Hart, E. B., *J. Nutrition*, 1948, v37, 263.

3. Reid, M. E., and Briggs, G. M., *ibid.*, 1953, v51, 341.

4. Heinicke, H. R., and Elvehjem, C. A., unpublished data.

5. Hegsted, D. M., Mills, R. C., Elvehjem, C. A., and Hart, E. B., *J. Biol. Chem.*, 1941, v138, 459.

6. Heinicke, H. R., Harper, A. E., and Elvehjem, C. A., *J. Nutrition*, in press.

7. Bixby, J. N., Bosch, A. J., Elvehjem, C. A., and Swanson, A. M., *Ag. and Food Chem.*, 1954, v2, 375.

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Hydrolysis of Succinylmonocholine by a Liver Esterase.* (21941)

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Succinylcholine (SDC), a short-acting muscle relaxant(1), has replaced to a considerable extent the older relaxants d-tubocurarine and decamethonium since its introduction in 1949(2). Little systematic work, however, has yet been reported on its mode of breakdown in the body. It is known(3) that there is little excretion of unchanged SDC after intravenous injection and that, *in vitro*, serum cholinesterase hydrolyses SDC to succinylmonocholine (SMC) and choline(4,5). This enzyme also hydrolyses SMC to succinic acid and choline, but at a much lower rate(5,6). In many patients with a low serum-cholinesterase level, a prolonged response to SDC is encountered(7,8) but it is also known that a similar abnormal prolongation of the period of relaxation after SDC administration can occur in subjects with normal serum-cholinesterase levels(9,10). It seems possible that this lack of correlation between serum-cholinesterase level and duration of relaxation may be due to the presence of another esterase which is also capable of hydrolysing SDC. It has also been suggested(11) that SMC, which

itself has slight muscle-relaxant properties (12), accumulates in the body after SDC administration and potentiates the action of SDC. Since the hydrolysis of SMC by serum cholinesterase is slow, especially in the presence of SDC(5), SMC might be expected to accumulate under these conditions unless there is another esterase in the body which is also capable of hydrolysing SMC.

It was therefore decided to determine whether esterases hydrolysing SDC or SMC are present in other tissues. The present communication concerns such an enzyme which is present in liver and which is distinct from liver cholinesterase. This enzyme catalyzes the hydrolysis of SMC but is without action on SDC.

Materials and methods. Samples of succinylcholine dichloride and succinylmonocholine iodide were kindly supplied by Burroughs-Wellcome & Co. The rats used were a local hooded strain (170-220 g). The animals were stunned and exsanguinated, and the tissues removed and placed in cracked ice. Homogenates (17% in water) were prepared in Potter-Elvehjem type homogenizers at 0-5°C. The rates of enzymatic and non-enzymatic ester hydrolysis were followed in

*We are most grateful to Messrs. Burroughs-Wellcome and Co., Inc., Tuckahoe, N. Y. for a grant which made this work possible.

TABLE I. Hydrolysis of SMC and SDC by Rat Liver Homogenate.

Substrate	Rate of hydrolysis, $\mu\text{l CO}_2/\text{hr}$ above endogenous		
	Without enzyme	With 170 mg homogenized rat liver	With 340 mg homogenized rat liver
.01M SMC	0	55	97
.02M "	1	54	110
.01M SDC	14	33	29
.02M "	28	58	58

the conventional Warburg apparatus by measurement of the CO_2 evolution from a 0.025 M sodium bicarbonate medium under anaerobic conditions at 37°C . The initial concentration of ester was normally 0.01 M, the total volume 3.0 ml and the vessels were gassed with 93% N_2 -7% CO_2 for 20 minutes. The substrates were tipped in from the sidearms and after a further period of 10 minutes an initial reading was taken. Readings were then taken every 10 minutes for a period of one hour. Liver homogenate was separated into sub-cellular fractions according to the method of Schneider (13).

Results. Rat liver homogenate increases the rate of production of CO_2 from SDC and, to a greater extent, from SMC. Unlike the hydrolysis by serum, this liver-catalysed hydrolysis was not inhibited by 10^{-3} M eserine or by 10^{-4} tetraethylpyrophosphate (TEPP). However, a study of the kinetics of SDC hydrolysis showed that the rate of hydrolysis was not increased when the concentration of liver homogenate was doubled, but that the rate was doubled by doubling the concentration of SDC (from 0.01 M to 0.02 M). With SMC, however, doubling the enzyme concentration doubled the rate of hydrolysis but increasing the substrate concentration from 0.01 M to 0.02 M gave no increase in activity. Typical results are given in Table I. Since SDC has an appreciable rate of non-enzymatic hydrolysis under the given experimental conditions, the enzymatic hydrolysis of the SMC produced by this process would account for the observed activity in the SDC-liver homogenate system. These observations led to the conclusion that the liver esterase hydrolyses SMC but not SDC. The enzyme is provisionally

termed "succinylmonocholine esterase" (SMC-esterase). The SMC-hydrolysing activities of rat, guinea pig, rabbit and human liver are shown in Table II.

Distribution of enzyme activity in rat liver. A 30% homogenate of rat liver in 0.25 M sucrose was fractionated by differential centrifugation at 0°C into nuclear, mitochondrial, microsomal and soluble fractions. As shown in Table III, most of the SMC-hydrolysing activity is located in the mitochondrial fraction. The slight activity observed in the nuclear fraction is probably due to the presence of some unbroken cells. In preliminary work on SMC-esterase, suspensions of guinea pig and rat liver mitochondria, prepared by fractional centrifugation in 0.25 M sucrose, were used as a source of the esterase. However, owing to the relative instability of these preparations, an attempt was made to prepare a stock of semi-purified enzyme in concentrated solution.

Preparation of a purified SMC-esterase. Acetone-dried rat liver was prepared by homogenizing rat liver with ice-cold acetone in a Waring blender, filtering by suction and washing several times on the filter with cold acetone. The powder was dried and stored in a vacuum desiccator at 0°C . The resulting powder contained an active SMC-esterase, but the enzyme could not be extracted from the powder with water. A suitable extraction medium was found to be 0.25 M ammonium

TABLE II. Hydrolysis of SMC by Liver Tissues.

Enzyme preparation	Rate of hydrolysis of 0.01M SMC, $\mu\text{l CO}_2/\text{hr}/100$ mg liver
Rat liver homogenate	30, 31, 33, 36
" " slices	34
Guinea pig liver homogenate	26, 26, 27, 28, 34
Rabbit liver homogenate	7, 8
Human liver homogenate (36 hr post-mortem)	13
Human liver homogenate* (fresh)	7

* This sample was removed as a small slice (250 mg) during a cholecystectomy operation and immediately frozen in an acetone-solid CO_2 mixture. Enzyme activity was determined 1 hr later. The low value may be due either to freezing procedure employed or to liver damage in the patient.

TABLE III. Hydrolysis of SMC by Cellular Fractions of Rat Liver.

Fraction	SMC-hydrolysing activity, $\mu\text{l CO}_2/\text{hr}/\text{mg nitrogen}^*$
Whole homogenate	10
Nuclear fraction	15
Mitochondria	130
Microsomes	5
Soluble	1

* Determined by micro-Kjeldahl method.

hydroxide. A 5% suspension of the rat liver powder in this medium was homogenized for 2 minutes in a Waring blender in the presence of a few drops of octyl alcohol. The suspension was allowed to stand at 5°C for 15 minutes and then centrifuged at 20,000 x gravity for 15 minutes. The viscous supernatant was brought to pH 7.4 with 5% acetic acid and the heavy precipitate formed was removed by centrifugation at 3,300 x gravity for 10 minutes. The resulting supernatant contained 60% of the total activity present in the original acetone powder. The extract was then fractionated with ammonium sulfate. The precipitate formed at 33% saturation with ammonium sulfate had only slight esterase activity with SMC as substrate, but when the concentration of ammonium sulfate was raised to 50% saturation, a precipitate was formed which contained 60% of the activity of the unfractionated extract. The supernatant from this precipitate contained no detectable activity. The activity of all fractions was determined after dialysis against running tap-water overnight.

The fraction with the high SMC-esterase activity (33-50% saturation) had a very low cholinesterase activity with acetyl- or benzoylcholine as substrate. Further, the SMC-esterase was unaffected by 10^{-4} M TEPP, whereas the cholinesterase was completely inhibited. Work is now in progress to determine the substrate specificity of the purified preparation of SMC-esterase and to study its resistance to other esterase inhibitors.

Discussion. SMC-esterase might prevent the accumulation of SMC in the body after SDC administration if present in an active form in man. This would suggest that SMC accumulation is not responsible for the prolonged effect of SDC sometimes observed.

However, it is possible that in such cases the liver SMC-esterase level and not the serum cholinesterase level is abnormally low and thus allows the accumulation of SMC. It is of interest that a choline ester, namely SMC, should be hydrolyzed in liver by an enzyme which is distinct from the cholinesterases of Whittaker's classification(14).

Summary. 1. The livers of rat and of other animals, including man, contain an esterase, distinct from known cholinesterases, which hydrolyses succinylmonocholine. The enzyme is located in the mitochondria and can be extracted from a liver acetone-powder with dilute ammonium hydroxide. It has been partially purified by ammonium sulfate fractionation of a liver extract, and the final preparation was almost free of cholinesterase activity. The enzyme is TEPP insensitive. 2. The possible role of this enzyme in recovery of muscular control after succinylcholine administration is discussed.

1. Bourne, J. G., Collier, H. O. J., and Somers, G. F., *Lancet*, 1952, v1, 1225.
2. Bovet, D., Bovet-Nitti, F., Guarino, S., Longo, V. G., and Marotta, M., *Rendiconti Ist. Sup. Sanita*, 1949, v12, 106.
3. Foldes, F. F., Vandervort, R. S., and Shanor, S. P., *Anesthesiology*, 1955, v16, 11.
4. Tsuji, F. J., and Foldes, F. F., *Fed. Proc.*, 1953, v12, 374.
5. Whittaker, V. P., and Wijesundera, S., *Biochem. J.*, 1952, v52, 475.
6. Foldes, F. F., *Proc. Soc. Exp. Biol. and Med.*, 1953, v83, 187.
7. Evans, F. T., Gray, P. W. S., Lehmann, H., and Silk, E., *Lancet*, 1952, v1, 1229.
8. Forbat, A., Lehmann, H., and Silk, E., *ibid.*, 1953, v1, 1067.
9. Borders, R. W., Stephen, C. R., Nowill, W. K., and Martin, R., *Anesthesiology*, 1955, v16, 401.
10. Hodges, R. H. J., and Harkness, J., *Brit. Med. J.*, 1954, v2, 18.
11. Foldes, F. F., McNall, P. G., and Birch, J. H., *ibid.*, 1954, v1, 967.
12. Ellis, C. H., Wnuck, A. L., Fanelli, R. V., and de Beer, E. J., *J. Pharmacol.*, 1953, v109, 83.
13. Schneider, W. C., *J. Biol. Chem.*, 1948, v176, 259.
14. Whittaker, V. P., *Physiol. Rev.*, 1951, v31, 312.

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Alterations in Renal Threshold for Sugar by Oral Administration of Cortisone Acetate. (1942)

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Because of widespread clinical use of ACTH and cortisone, their influence upon renal threshold for sugar is of more than academic interest. Holten and Lundbraek(1) noted glycosuria without appreciable rise in fasting or maximum blood sugar levels in well controlled patients by administration of 100 mg of ACTH daily. These findings were confirmed by Kass(2), whereas Fillipis(3), working independently, found the renal threshold for glucose to be significantly increased after use of ACTH in single doses not higher than 40 mg. The effect of DOC glucoside on renal tubular reabsorption in dogs was investigated by Despopoulos and Kaufman(4), who found a reversible depression of renal reabsorption capacity for glucose. To obtain such data after the use of cortisone, renal threshold determinations were made in 8 patients.

Methods and procedures. Four patients were studied, 2 men and 2 women, aged 75, 77, 59, and 64, respectively. One had a diaphragmatic hernia and no other pathology. The other 3 were objects of custodial care without any obvious pathologic conditions, except a degree of arteriosclerosis compatible with age. None of the patients had ever had evidence of diabetes mellitus. After 10 P.M. of the evening preceding each test, food and fluids were withheld. An indwelling catheter was inserted 15 minutes before the test and the bladder emptied. After a fasting blood sugar specimen had been taken, infusion of a 20% solution of glucose in water was started. The infusion was allowed to flow at a speed of 10 ml per minute. The blood and urine were examined for glucose at 5-minute intervals, the infusion stopped as soon as a positive reaction for sugar was found in the urine. For each of 6 immediately consecutive days, the patients were given 200 mg of cortisone acetate orally and their regular ward diets

were kept at an isocaloric level of 2200 calories. On the 7th day, the above procedure was repeated. Before, during and after the test, patients were on a regular ward diet. This method provided us with some rather strikingly uniform data about the "breakdown" of the kidney threshold, as a result of administration of glucose and glucose in conjunction with cortisone acetate. In a second group of 4 patients, we determined not only the point at which the renal threshold was exceeded, but also the point of its restoration after treatment with glucose alone and glucose plus cortisone acetate, respectively. The technic remained essentially the same as for the first study, except that values for glucose in blood and urine were determined after stopping the infusion until the urine was found to be aglycosuric. The subjects of the second group, 2 of whom were men, 69 and 76 years old, respectively, and 2, women, 70 and 72 years old, respectively, were free of any active organic disease, other than the vascular changes compatible with age.

Results. Study I. In all 4 patients of the first group, we found a lowered threshold for glucose following treatment with cortisone. Glycosuria occurred at definitely lower level for blood sugar. The difference between values before and after cortisone varied from 52 to 132 mg per 100 ml (Table I and Fig. 1). The average decrease in the threshold for glucose was 86 mg per 100 ml. Administration of cortisone was associated with a shortening of the time necessary to exceed the renal threshold for glucose in 2 subjects, a lengthening in one, and no change in the fourth (Table I).

Study II. In the second group of 4 patients, we confirmed the findings of Study I, although in case No. 7 (Table I) the decrease in the threshold was not as evident as in the other subjects. In this individual, during the first test, glucose appeared in the urine between

*Wakefield Foundation Fellow in Research.

TABLE I. Influence of Cortisone on Renal Threshold for Glucose in 8 Normally Aging Subjects. Determinations of glucose in blood (mg/100 cc) and urine at periodic intervals (min.).

Pt. No.	Age (yr)	Sex	Specimen	Before and after treatment with cortisone															
				0		5		10		15		20		25		30		35	
				B*	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A
1	75	♀	Blood	98	120	206	253	240	364	416	478	560	544	630					
			Urine	0	0	0	0	0	1+	1+	2+	2+	3+	3+					
2	77	♀	Blood	101	89	236	253	369	305	437	358	518	415						
			Urine	0	0	0	0	0	3+	3+	3+	4+	4+						
3	59	♂	Blood	142	115	229	183	370	248	434	287	443	327	495	334	438			
			Urine	0	0	0	0	0	0	0	0	2+	0	3+	1+	4+			
4	64	♂	Blood	99	66	144	152	234	166	282	220	344	290	368	302				
			Urine	0	0	0	0	0	0	1+	1+	3+	3+	4+	4+				
5	69	♂	Blood	102	100	205	187	306	322	347	330	395	372						
			Urine	0	0	0	0	0	0	2+	2+	4+	4+						
6	76	♂	Blood	121	90	334	281	501	346	532		451							
			Urine	0	—	0	—	2+	1+	3+		4+							
7	70	♀	Blood	70	91	220	218	327	358	346									
			Urine	0	0	0	0	0	3+	3+									
8	72	♀	Blood	122	108	154	172	196	311	345	370	428	403	512					
			Urine	0	0	0	0	0	1+	2+	3+	3+	4+	4+					

* B = Before; A = After.

Solid underline shows point of break in tolerance. Dotted underline shows point of restoration of tolerance.

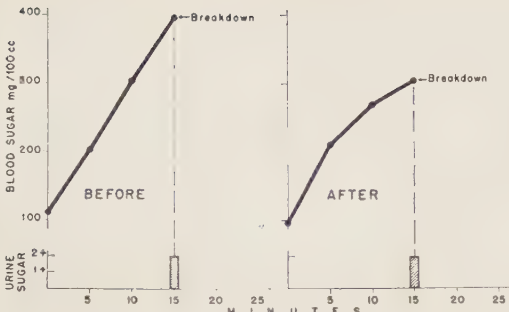


FIG. 1. Breakdown of the renal threshold for sugar before and after treatment with cortisone acetate—200 mg orally daily for 6 days (arithmetical averages for cases 1-4).

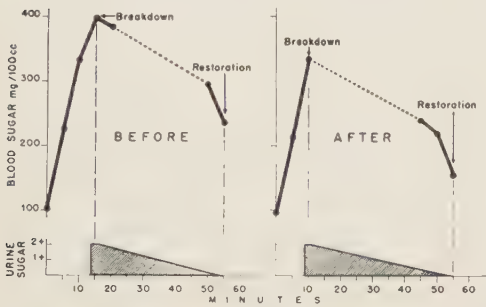


FIG. 2. Breakdown and restoration of the renal threshold for sugar before and after treatment with cortisone acetate—200 mg orally daily for 6 days (arithmetical averages for cases 5-8).

blood sugar levels of 327 and 346 mg per 100 ml, while after administration of cortisone acetate this occurred between 218 and 358 mg per ml. During the 5-minute span, it seems likely that glucose may have been excreted into the bladder before the higher level of 358 mg per 100 ml was reached.

In addition to confirmation of the previous findings, we determined the "restoration" of the kidney threshold in these 4 subjects. In all of them, there was a marked decrease in the blood sugar level at which restoration of the threshold occurred following the use of cortisone acetate (Table I and Fig. 2). The dif-

ferences before and after the use of cortisone acetate varied from 35 to 116 mg per 100 ml with an average of 89 mg per 100 ml. Not only was the renal threshold for sugar lowered by cortisone, but also all values for blood sugar seemed to be depressed, thus producing a depressed or low glucose tolerance curve (Fig. 2). Following administration of cortisone acetate, the time necessary to exceed the renal threshold for sugar was unchanged in 2 and shortened in 2 (Table I). The time required to "restore" the renal threshold for sugar was not altered by the amounts of cortisone given in these studies (Table I and Fig. 2).

Summary. A simple method is described for determination of the renal threshold for glucose in untreated and cortisone-treated individuals. In 4 patients the "breakdown" of the glucose-threshold was investigated and was found to be markedly decreased after 6 days of orally administered cortisone, 200 mg daily. The decrease amounted to an average of 86 mg per 100 ml of blood with a range from 52 to 132 mg per 100 ml. In another group of 4 patients, both the "breakdown" and the "restoration" thresholds were determined. The breakdown values for the first group were confirmed and in addition the restoration of the threshold was also found to be at a lower level for blood sugar, averaging 68 mg per 100 ml less after cortisone than before it with individual variations from 35 to 116 mg per 100 ml.

1. Holtén, C., and Lundbraek, K., *Scandinav. Clin. and Lab. Invest.*, 1950, v2, 317.
2. De Filippis, V., *Lancet*, 1951, v1, 856.
3. Kass, E. H., *PROC. SOC. EXP. BIOL. AND MED.*, 1950, v73, 669.
4. Despopoulos, A., and Kaufman, E. H., *Am. J. Physiol.*, 1952, v170, 11.

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Suppression of Adrenal Compensatory Hypertrophy by Hypothalamic Lesions.* (21943)

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There is general agreement that lesions of the median eminence suppress the acute adrenal cortical response to stress(1,2,3). Interruption of the supraopticohypophyseal tract as evidenced by diabetes insipidus appears to be responsible for this failure of the normal stress-induced discharge of adrenocorticotrophin (ACTH)(4). In the adrenalectomized rat maintained on desoxycorticosterone, acute stress results in very high levels of blood ACTH. If rats with hypothalamic lesions are adrenalectomized and subjected to acute stress, no detectable ACTH can be found in blood(5). In addition to acute stress, it is known that adrenalectomy results in an increase in pituitary ACTH output(6,7). This increase can not be demonstrated in adrenalectomized rats with hypothalamic lesions, and this suggests that the increased ACTH secretion following adrenalectomy may also be dependent upon hypothalamic activity(5). However, small increases in blood ACTH concentration above the normal, unstressed level would not be detected by the assay employed. It was of interest, therefore, to determine whether the compensatory hypertrophy which follows unilateral adrenalectomy would occur in rats with hypothalamic lesions. This hypertrophy is presumably due also to increased ACTH discharge consequent to a fall in blood steroids following unilateral adrenalectomy(8,9). The present results indicate that lesions which interrupt the supraopticohypophyseal tract suppress the compensatory hypertrophy following unilateral adrenalectomy, but they also suggest that a small response may be present.

Methods. Bilateral lesions were produced in the hypothalamus of adult male rats of the Wistar strain with the aid of a Krieg stereotaxic instrument. Three weeks to 2 months postoperatively, the rats were subjected to

left unilateral adrenalectomy under ether anesthesia. Two weeks later the right adrenal was removed. Control rats without lesions were similarly studied. Adrenals were weighed to the nearest 0.1 mg on a torsion balance. Because of variations in initial adrenal weights, all results are expressed as the percentage change in right adrenal weight after unilateral adrenalectomy as compared to the weight of the previously removed left adrenal.

Results. Twelve normal rats subjected to unilateral left adrenalectomy exhibited an hypertrophy of the right adrenal equal to $51 \pm 8\%$, when it was removed 2 weeks later. Since previous work has shown that the blockade in the acute adrenal stress response in rats with lesions correlates well with the degree of interruption of the supraopticohypophyseal tract as judged by increased water intake(4), the compensatory adrenal hypertrophy of the control rats and of 48 rats with lesions of the hypothalamus was plotted against their daily water intake in Fig. 1. It

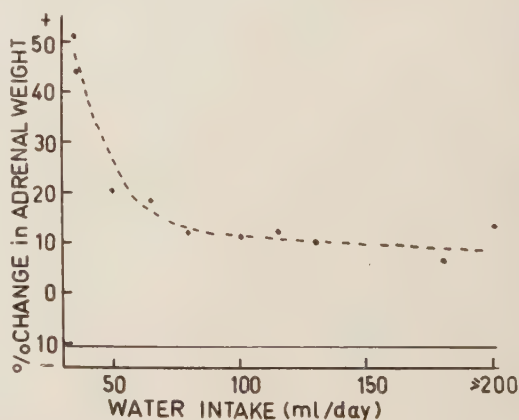


FIG. 1. Suppression of compensatory adrenal hypertrophy in rats with diabetes insipidus. Daily water intake on abscissa is plotted against percentage change in right adrenal wt. Each point represents mean response of 4 or more rats for each increment of 10 ml or a multiple thereof of water consumption per day. Solid line represents result found in rats with lesions where both adrenals were removed simultaneously.

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can be seen that the degree of hypertrophy fell off very rapidly as water intake increased above the normal level of 35 ml/day. At levels of water intake above 100 ml/day, the compensatory hypertrophy fell to a minimal value of approximately 10%, and it remained at this figure with increasing values of water intake. The mean increase in right adrenal weight of all 20 animals with water intakes of 100 ml/day or more was $+9 \pm 4\%$. In another series of rats with hypothalamic lesions, when the 2 adrenals were removed simultaneously, the right adrenal weighed on the average $11 \pm 2\%$ less than the left adrenal. Consequently, in the rats with diabetes insipidus the response to unilateral adrenalectomy was $+9 \pm 4\%$ plus $11 \pm 2\%$, or a total gain of approximately 20% above the expected weight of glands from this location in rats with lesions. This gain is 30% of the gain exhibited by normal animals. This small residual response was statistically significant if compared to the results obtained when both adrenals were removed simultaneously in the rats with lesions ($P < 0.01$).

Nearly all rats gained weight during the experimental period. Five of the 53 rats with lesions, however, lost 10% or more of their body weight during this time, and all of these 5 animals exhibited hypertrophy of the remaining adrenal, the mean response being $+56 \pm 14\%$, a value similar to that of the control rats without lesions. This hypertrophy in rats with weight loss was independent of the water intake which varied between 90 and 380 ml/day. The results in these rats were excluded from consideration in Fig. 1.

Testicular atrophy occurred in only 6 of the 52 rats studied, a result in harmony with previous studies indicating that suppression of adrenal function can occur in the absence of testicular atrophy(1,5).

Discussion. The present results indicate that compensatory adrenal hypertrophy is suppressed in rats with hypothalamic lesions. This is interpreted to mean that such lesions at least partially block the augmented secretion of pituitary ACTH which normally ensues when the blood level of corticoids is lowered as a result of unilateral adrenalectomy. A small response still occurs in such rats.

Furthermore, if the animals lose significant weight during the course of the study, they still manifest compensatory hypertrophy. This hypertrophy is presumed to be due to some additional stress which appears to be capable of evoking a discharge of ACTH when acting over this 2-week time interval even in the rat with hypothalamic lesions. It has been previously demonstrated that rats with extremely severe diabetes insipidus show adrenal hypertrophy although the acute adrenal stress response is blocked(4,5). Sufficient rats with severe diabetes insipidus have not been subjected to unilateral adrenalectomy to allow conclusions as to whether they would still respond with compensatory hypertrophy.

The lesions in the present study were designed to interrupt the supraopticohypophyseal tract in the rostral portion of the median eminence. Damage to other structures undoubtedly occurred, but the correlation between diabetes insipidus and reduction in the response to unilateral adrenalectomy agrees with the results of previous work in which it was demonstrated that blockade of the acute adrenal stress response in rats could be correlated only with injury to the supraopticohypophyseal tract(4).

Our results are in good agreement with those recently reported by Ganong and Hume in the dog(10), who correlated a complete absence of compensatory hypertrophy with lesions which destroyed an estimated 50% or more of the median eminence. In our experience a small response is still present in rats with lesions.

The secretion of ACTH which normally follows adrenalectomy is believed to be caused by the fall in blood level of corticoids following removal of the adrenals(5,7). In animals with appropriate hypothalamic lesions there is a suppression of this ACTH secretion. It appears that this suppression may have two possible explanations. Either the steroids normally act directly upon the hypothalamic or other nervous structures to inhibit neural activity when the blood level is raised and to stimulate it when the level falls. Or if the steroids act directly upon the cells of the anterior pituitary gland, then the sensitivity of

these cells to the level of cortical steroids present in the blood must be affected by the neurohumor presumed to be secreted by the hypothalamus. Our data do not permit us to decide which of these possibilities is correct.

Conclusions. The compensatory adrenal hypertrophy which normally follows unilateral adrenalectomy is suppressed in rats with diabetes insipidus produced by hypothalamic lesions.

1. McCann, S. M., *Am. J. Physiol.*, 1952, v171, 746.
2. Hume, D. M., *Ann. Surg.*, 1953, v138, 548.
3. Anand, B. K., Raghunath, P., Dua, S., and Mohindra, S., *Indian J. Med. Research*, 1954, v42, 2.

4. McCann, S. M., and Brobeck, J. R., *PROC. SOC. EXP. BIOL. AND MED.*, 1954, v87, 318.
5. McCann, S. M., and Sydnor, K. L., *ibid.*, 1954, v87, 369.
6. Gemzell, C. A., Van Dyke, D. C., Tobias, C. A., and Evans, H. M., *Endocrinol.*, 1951, v49, 325.
7. Sydnor, K. L., and Sayers, G., *ibid.*, 1954, v55, 621.
8. Tepperman, J., Engel, F. L., and Long, C. N. H., *ibid.*, 1943, v32, 373.
9. Ganong, W. F., *ibid.*, 1954, v55, 117.
10. Ganong, W. F., and Hume, D. M., *ibid.*, 1954, v55, 474.

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Plaque Formation of Poliomyelitis Viruses on Human Amnion Cell Cultures.* (21944)

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In a previous publication(1) human amnion cells were reported to support propagation of poliomyelitis viruses, and it was indicated that tissue cultures obtained from this readily available tissue could provide a source for the large scale production of poliomyelitis virus.

In the present work plaque formation in monolayer tissue cultures of amnion cells by the 3 types of this virus was obtained, similar to the plaque formation by this virus in monkey kidney and testis cultures(2) and human cancer (HeLa) cell cultures(3). It is, therefore, possible to employ this tissue also for the quantitative assay of poliomyelitis virus.

Material and methods. *Virus.* Poliomyelitis viruses, type 1, strain Mahoney; type 2, strain MEF-1; type 3, strain Saukett, obtained from Dr. Jonas Salk in 1952, were passed several times in monkey kidney cells, 3-4 times in HeLa cells and finally 1 or 2 times in monkey kidney cells. The final virus preparations were stored at -60°C . *Cells.* Monolayer cultures of human amnion cells

were prepared in 50 mm petri dishes as previously described(1). After centrifugation the cell pack was diluted 1:120 and the cultures were grown at 37°C in a 3% CO_2 -atmosphere in a medium consisting of 20% ox serum and Earle's balanced salt solution containing 0.5% lactalbumin hydrolysate (Nutritional Biochemicals). The culture fluid was replaced by fresh medium after incubation for 4-5 days. The cell sheet was usually confluent after 10-14 days. *Infection and overlay.* The cultures were washed 3 times with buffer solution at 37°C after removal of the nutrient fluid and inoculated with 0.3 ml of various dilutions of the virus preparation. After permitting adsorption of the virus for at least 30 minutes at 37°C , the plates were overlayed with 3-4 ml of melted agar containing nutrient fluid. 100 ml nutrient fluid consisted of 55 ml 1% Difco yeast extract in distilled water, 6 ml 5% bovine albumin powder V (Armour) in Hanks' solution, 9 ml 5% NaHCO_3 in distilled water and 30 ml 10 times concentrated Hanks' solution without phenol red(4). One part of 3% agar was melted and kept in a water bath at 44°C . One part of the nutrient fluid was mixed with an equal volume of dis-

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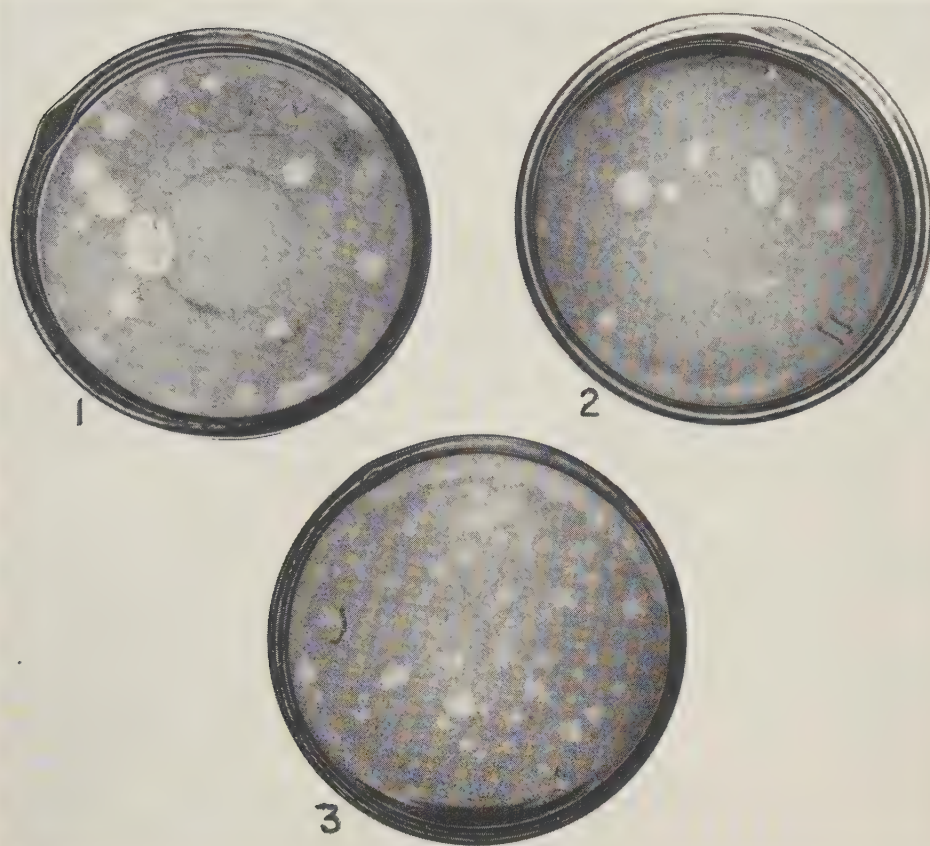


FIG. 1. Plaques of poliomyelitis viruses on human amnion cell cultures 72 hr after infection.
1: Type 1, strain Mahoney; 2: Type 2, strain MEF-1; 3: Type 3, strain Saukett.

tilled water and added to the agar. After $2\frac{1}{2}$ days of incubation at 37°C the plates were stained with 0.3 ml of 1:4000 neutral red in phosphate buffer solution.

The monkey kidney plate cultures used for comparative assays were prepared by the technique devised by Dulbecco and Vogt(2) and grown in a medium consisting of 2% ox serum and Earle's balanced salt solution with 0.5% lactalbumin hydrolysate. The plates were infected, overlayed and incubated as were the amnion plates.

Results. Fig. 1 shows the plaques produced by inoculating suitable dilutions of the 3 types of poliomyelitis virus on monolayer cultures of first generation human amniotic cells. The plaques were almost round areas which could be easily seen and counted without magnification. The size of the plaques

was somewhat variable and increased with time of incubation until the plaques became confluent. At an early time when the plaques were still small it was possible to count up to approximately 200 plaques in one plate, since they were very easily discernible.

Table I shows a comparison of titers obtained for 4 different samples assayed on monkey kidney and human amnion plate cultures, at the same time under identical conditions. It can be seen that the number of plaque forming units per ml of Mahoney and MEF-1 virus estimated from the amnion plates was consistently higher than that from the monkey kidney plates. This difference was not observed for the single Saukett virus assay. More detailed comparison of the titers obtained in the 2 tissue culture systems is being made in this laboratory.

TABLE I. Number of Plaques and Calculated Plaque Forming Units (PFU) per ml for 4 Samples of Poliomyelitis Virus Assayed on Monkey Kidney and Human Amnion Plates in Various Dilutions.

Virus samples	Dilution	Cell layer from			
		Monkey kidney		Human amnion	
		Plaques/plate	PFU/ml	Plaques/plate	PFU/ml
Mahoney	$10^{-5.5}$	19, 20, 21, 19	2.1×10^7		
	10^{-6}	9, 7, 5, 5	2.2×10^7	25, 24	8.2×10^7
	10^{-7}			0, 6	1.0×10^8
"	10^{-6}	38, 41, 43	1.4×10^8		
	$10^{-6.5}$	24, 17, 15	2.0×10^8		
	10^{-7}	2, 5, 4	1.2×10^8	26, 26	8.7×10^8
	$10^{-7.5}$			8, 11, 11	1.1×10^9
MEF-1	10^{-6}	25, 25, 34	8.9×10^7	113, 115	3.8×10^8
	$10^{-6.5}$	10, 10, 7	9.6×10^7		
	10^{-7}	2, 4, 3	1.0×10^8	10, 4	2.3×10^8
Saukett	10^{-4}			217, 189	6.8×10^9
	10^{-5}	88, 107, 90	3.2×10^7	85, 68	2.6×10^7
	$10^{-5.5}$	31, 32, 31	3.3×10^7		
	10^{-6}	3, 11	2.3×10^7	3, 5	1.3×10^7

Summary. Plaque formation of all 3 types of poliomyelitis virus has been obtained in monolayer cultures of human amnion cells. The plaque titer obtained on amnion was 3-6 times higher than on monkey kidney cell cultures for strains Mahoney and MEF-1.

Thelma H., *Science*, 1955, v122, 30.

2. Dulbecco, R., and Vogt, Marguerite, *J. Exp. Med.*, 1954, v99, 167.

3. McClain, Mary E., and Schwerdt, C. E., *Fed. Proc.*, 1954, v15, 505, abstract.

4. Youngner, J. S., 1955, personal communication.

1. Zitcer, Elsa M., Fogh, J., and Dunnebacke,

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Propagation of Newcastle Disease Virus in Ehrlich Ascites Cells *In vitro* and *In vivo*.* (21945)

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(Introduced by Herald R. Cox.)

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Mengo encephalitis virus has been cultivated in the Ehrlich ascites carcinoma cell *in vitro* (1). While no cytopathogenic effect of Mengo virus—in spite of its multiplication—was visible in the test tube, its oncolytic property was readily demonstrated in the ascites cells *in vivo*. Some workers have reported the inhibition of Ehrlich ascites tumor growth when the inoculum was first mixed with Newcastle disease virus (NDV) either just prior to or at the time of inoculation (2). However,

sustained multiplication of the NDV in the Ehrlich cells could not be demonstrated (3).

It seemed of interest to ascertain whether this virus could be adapted to grow in the Ehrlich ascites tumor and to determine whether viral multiplication would result in oncolysis *in vivo*.

Material and methods. Virus. The Newcastle disease virus was originally isolated in Massachusetts in 1945 by Dr. Van Roekel, and is designated MD20Z. It was received by Dr. Floyd Markham from the United States Bureau of Animal Industry as the fifth chick embryo passage of the original strain.

* The authors wish to thank Dr. Hilary Koprowski for his helpful suggestions, and Donald Hendrickson for his valuable technical assistance.

In this laboratory it was passaged once in chick embryos, then 12 times in duck embryos, then twice in chick embryos. The present designation of MD20Z indicates the total passages of the virus to be 20. Undiluted allantoic fluids make up the virus pool. *Tumor.* The Ehrlich ascites tumor was received through the kindness of Dr. George Klein of the Karolinska Institute, Stockholm, Sweden. The tumor has been continuously passaged in this laboratory since 1950 in Swiss (or ICR) mice every 7 days. In order to test the effect of the virus preparations *in vivo*, female Swiss mice weighing 20-25 g were inoculated i.p. with 0.2 ml of pooled ascitic fluid containing 10-30 million tumor cells. *Virus-tumor cell suspension.* Ascites cells were collected 7-10 days after implantation, washed as previously described(1), resuspended in an arbitrary volume of Parker's synthetic medium 199(4) and the number of cells was counted in a standard bright-line Neubauer hemocytometer. The average of 4 counts was taken in order to determine the cell concentration. In all experiments the final concentration of Ehrlich ascites cells was adjusted with medium 199 to give 5×10^6 cells per ml. Penicillin and streptomycin were routinely added to the culture medium to give a final concentration of 100 units and 200 γ per ml respectively. Virus was added as noted in each experiment. Two ml aliquots of the cell-virus suspensions were transferred to 15 x 150 mm pyrex test tubes, which were closed with silicone stoppers, placed in a roller drum and incubated at 37°C for the desired time interval. After incubation the cultures were harvested, pooled and stored in rubber-stoppered vaccine bottles at -25°C until the virus was used in the next tissue culture passage or egg titration was performed. *Egg titrations.* Tenfold dilutions of tissue culture or ascites fluid were made in 2% N-Z[†] amine solution (Lederle) containing 2 mg of streptomycin per ml. This material was then injected in 0.2 ml amounts into the chorioallantoic cavities of 9-11-day-old embryonated chicken eggs. Eggs were incubated at 37°C, candled

daily for seven days, and the number of dead embryos recorded. LD₅₀ titers were calculated according to the method of Reed and Muench(5). *Virus identification.* Newcastle immune chicken serum of high titer, prepared in this laboratory, was diluted 1:2 with 2% N-Z amine solution containing 2 mg of streptomycin per ml; mixed with an equal volume of undiluted ascitic or tissue culture fluid and with 10-fold dilutions thereof; and incubated at room temperature for 60 minutes. Eggs were inoculated in groups of 5, incubated at 37°C, and candled as described above. Virus identification was confirmed when protection was afforded by the immune serum mixture in all the dilutions through 10° and no protection was afforded by normal serum in control egg inoculations performed simultaneously. *Mouse inoculation.* At frequent intervals aliquots of 0.5 ml of undiluted tissue culture passage material were inoculated i.p. into a number of Swiss mice which had received transplants of the Ehrlich ascites tumor 4 days earlier. A control group of tumor-bearing mice was given a similar inoculum of uninfected tissue culture material prepared in the same manner and at the same time as the infected cultures. *Analysis of oncolytic effect and viral growth.* Smears of the ascitic fluid from at least 2 experimental and control mice were prepared daily, stained by Leishman's method, and examined. Karyorrhexis of tumor cells or their disappearance from the fluid was regarded as cytological evidence of oncolysis(6). This assessment of oncolysis was confirmed by determination of the survival

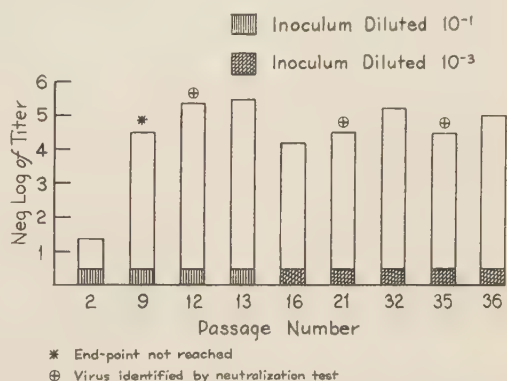


FIG. 1. Egg infectivity titer of NDV-Ehrlich cell tissue culture passages.

[†] Tryptic digest of casein (Sheffield Chemical Co., N. Y. City) 2% solution in distilled water.

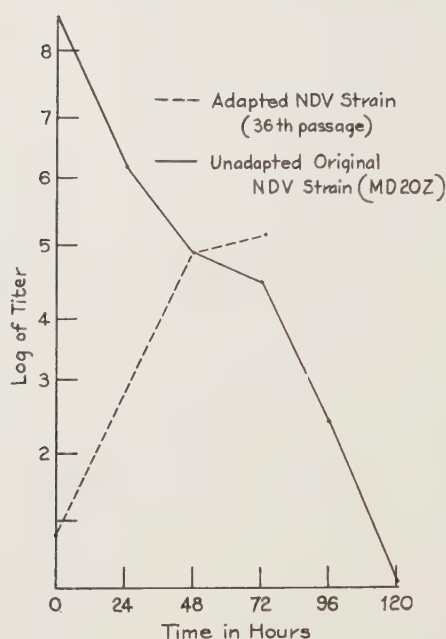


FIG. 2. Virus titer of NDV in Ehrlich cell cultures* after various intervals of incubation.

* Final dilution of MD20Z = 10^{-1} in 5×10^6 Ehrlich cells/ml.

ratio of experimental and control groups over a period of 30 days. In certain experiments 3 to 5 mice were killed, the ascitic fluid pooled, and egg infectivity titrations performed. In some instances neutralization tests were also done.

Results. Virus passage. The serial passage of NDV in the Ehrlich ascites cells *in vitro*

was begun with a ratio of one egg LD₅₀ of the MD20Z strain to 10,000 cells. After 96 hours incubation, the tube contents were harvested and pooled. This pool, in a final dilution of 10^{-1} , served as inoculum for the second passage, which was incubated as before. Starting with the third passage and continuing to the present (38th), incubation has been for 48 hours only. From the 13th passage on, each tissue culture inoculum was a 10^{-3} dilution of the preceding tissue culture passage.

Virus titers at various tissue culture passage levels. Egg infectivity titers were determined at various passage levels. Fig. 1 gives the results of representative titrations, and reveals the uniformity of infectious virus content at levels after the second passage. Thus the 13th and 16th passages contained approximately the same amounts of virus as subsequent passages, although the latter received smaller inocula. Fig. 2 shows the increase, during a period of 48 hours, in egg infectious virus in the 36th tissue culture passage. In contrast, a rapid drop in titer occurred when the unadapted parent NDV strain was inoculated into Ehrlich cell cultures. With the adapted strain there was an increase of approximately 4 logs in the period of 48 hours, whereas the drop in infectivity titer of the unadapted strain over the same period of incubation was 3.5 logs, and no virus was detectable after 120 hours.

Inoculation of ascites-bearing mice with

TABLE I. Summary of Experiments on Ascites-Bearing Mice Inoculated with Newcastle Disease Virus Tissue Culture.

Passage No.	Cytological evidence of oncolysis	Onset of karyorrhexis (days after inoculation of virus)	Survival ratio*	Virus titer after 96 hr
Parent strain	0	—		0
5	0	—	0/20	10^{-4}
10	+	7	0/20	$<10^{-4}$
12	0	—	0/20	$<10^{-4}$
18	+	7	2/20	N.D.
28	++	3	4/20	N.D.
29	+++	3	14/33	$10^{-6.0}$
37	+++	4	14/22	$10^{-7.5}$

* No. of mice surviving over No. of tumor-bearing mice.

N.D. = Not done.

All 150 control mice which did not receive virus inoculum died.

+ = Karyorrhexis, but tumor cells persisted until death. ++ = Complete disappearance of tumor cells in 50% of mice. +++ = Complete disappearance of tumor cells in all mice (see footnote ‡).

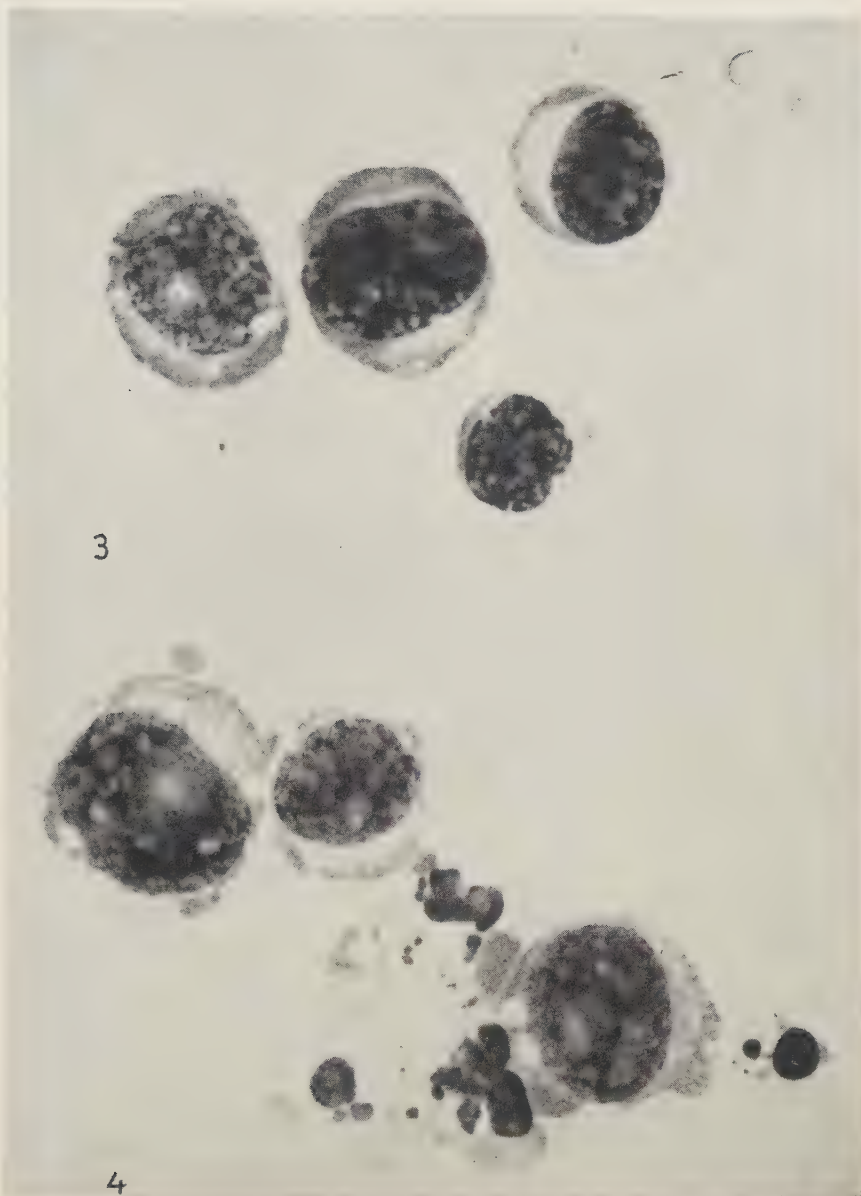


FIG. 3. Ehrlich ascites tumor cells 7 days after inoculation of uninfected tissue culture material. Leishman's stain $\times 1400$.

FIG. 4. Karyorrhexis in 2 Ehrlich ascites tumor cells 7 days after inoculation of 10th passage of NDV in tissue culture. Leishman's stain $\times 1400$.

NDV tissue culture. Virus from 7 different tissue culture passages was injected into mice bearing 4-day ascites tumors. Table I gives a summary of these experiments, which indicated that tissue culture passage of the Ehrlich-adapted strain of NDV enhanced its ability to produce oncolysis. Initially no on-

colysis was evident, and it was not until the 10th passage that karyorrhexis was observed. This occurred 7 days after virus inoculation (Fig. 3 and 4). Inocula from later tissue culture passages produced more effective and more rapid oncolysis, and the survival ratios increased (Table I). Karyorrhexis was ob-

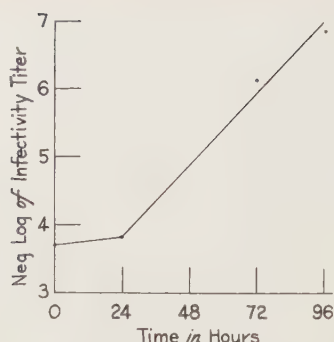


FIG. 5. Increase in egg infectivity titers of ascitic fluid of mice injected with adapted NDV.*

* 29th tissue culture passage.

served 4 days after injection of virus from the 37th tissue culture passage; no tumor cells were seen in the fluid withdrawn from any of the 4 mice on the 7th day after virus inoculation,† and the survival ratio was 14/22. No distinction was made between death from ascites and later death from solid tumors at the site of the original tumor injection. No virus could be detected in ascitic fluid obtained 96 hours after injection of the parent NDV strain; after 29 passages of the virus *in vitro* a comparable sample of ascitic fluid titrated to $10^{-6.8}$ in eggs. Fig. 5 shows a 3.5 log increase in egg-infectivity titer of the ascitic fluids obtained daily for 96 hours after injection of virus from the 29th tissue culture passage.

Virus identification. Undiluted tissue culture material from the 12th, 21st and 35th passages, as well as undiluted ascitic fluid obtained from mice 96 hours after injection with the 29th and 36th passages, was completely neutralized by NDV immune serum. Also chorioallantoic fluids withdrawn from embryonated chicken eggs injected with NDV-normal serum controls were spot tested, and gave positive hemagglutination which was specifically inhibited by high titer NDV immune serum.

Discussion. Inhibition of growth of the

† Less than 1% of tumor cells was observed in one smear, but most of these showed advanced degenerative changes.

Ehrlich ascites tumor by the tissue culture adapted strain of NDV is different from the phenomenon described by Moore and Diamond(2). These workers regarded the effect of the virus on tumor cells as comparable to the hemagglutinating action of viruses on red cells(6). They did not observe any cytological evidence of oncolysis like that described above. The cytological changes which follow inoculation of the tissue culture adapted NDV are essentially the same as those which have been described during the destruction of Ehrlich tumor cells after infection with West Nile and Bunyamwera viruses(6,7). Since the latter are not hemagglutinating viruses, the cytolytic action of the adapted strain of NDV is probably of the same nature as that of other oncolytic viruses, and like them depends upon the multiplication of virus in the tumor. This was confirmed by the absence of cytological changes after inoculation of the original strain of NDV, when virus multiplication in the tumor could not be demonstrated; indeed, only the later passages of the adapted virus which multiplied to a high degree were capable of inducing consistent oncolysis.

Summary. The propagation of NDV in Ehrlich ascites tumor cells maintained *in vitro* is described. After prolonged serial passages *in vitro*, multiplication of the virus occurs in the tumor *in vivo*, and in the later passage levels is accompanied by cytological and biological evidence of oncolysis.

1. Flanagan, A. D., and Colter, J., *Cancer Res.*, in press.
2. Moore, A. E., and Diamond, L. C., *J. Immunol.*, 1953, v71, 441.
3. ———, *Proc. Soc. Exp. Biol. and Med.*, 1952, v79, 663.
4. Morgan, J. R., Morton, H. J., and Parker, R. C., *ibid.*, 1950, v73, 1.
5. Reed, L. J., and Muench, H., *Am. J. Hyg.*, 1938, v27, 493.
6. Love, R., Koprowski, H., and Cox, H. R., *Cancer Res.*, 1953, v13, 350.
7. Orsi, E. V., Love, R., and Koprowski, H., *Proc. Am. Assn. Cancer Res.*, 1955, v2, 38.

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Susceptibility to Poliomyelitis Virus of HeLa Cells Adapted to Growth in Horse Serum Medium. (21946)

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With the development of a subline of HeLa cells capable of growth in medium containing horse serum in place of human serum(1) it was of interest to determine its susceptibility to the effects of poliomyelitis virus.

Materials and methods. HeLa cells adapted to growth in human serum and the subline grown in horse serum were obtained from Dr. Virginia Evans in Dr. Earle's laboratory, National Cancer Institute, in the form of flask cultures on glass. Monkey kidney cultures were prepared according to the trypsinization method of Youngner(2). HeLa cells of both sublines were transferred from large bottles into tubes by mechanical washing of cells off glass with the proper growth media. 0.5 ml of a suspension containing approximately 75,000 cells per ml was inoculated into each tube. The tubes were incubated for 24-48 hours in a stationary position on their sides before use in the tests with virus. The growth medium consisted of 40% Earle's balanced salt solution, 20% ultrafiltrate from chick embryo extract, and 40% serum. At the end of the short growth period these cultures were changed to Eagle's synthetic medium containing 4% horse serum(3), which was quite efficient as a maintenance medium for both HeLa cell lines. Monkey kidney cultures were prepared in roller tubes by growing the original trypsinized kidney suspension in Melnick's "A" medium(4) for 4 days, at which time they were changed to a maintenance medium consisting of 75% Earle's balanced salt solution, 24% ox serum ultrafiltrate, and 1% horse serum. Poliomyelitis viruses used for quantitating the susceptibility of the cell lines were the Mahoney Type I, 6468 Type II (isolated in this laboratory from the stool of a case of paralytic polio), and the Saukett

TABLE I. Titrations of Standard Pools of 3 Types of Poliomyelitis Virus in Monkey Kidney Cells and HeLa Cells Grown in Horse Serum and Human Serum Media.

Tissue culture	Poliomyelitis virus type					
	Exp. 1			Exp. 2		
	I	II	III	I	II	III
HeLa						
(Human serum)	5.75*	4.5	5.75	5.75	5.75	5.75
(Horse ")	4.75	3.5	3.75	6.5	5.25	6.25
Monkey kidney	5.75	5.75	5.75	6.5	6.25	6.25

* Expressed as logs of 50% end-points of titrations per 0.1 ml.

strain of Type III. Titrations of standard virus pools were carried out by inoculating 0.1 ml of each serial 10-fold dilution into each of 3 tissue culture tubes. Dilutions were carried out in Hanks' balanced salt solution. Cultures were read daily by microscopic examination for at least 7 days after inoculation. Stool emulsions prepared by extraction with balanced salt solution containing 2500 units each of penicillin and streptomycin, and clarified by centrifugation at 8000 r.p.m. for 30 minutes in a Spinco centrifuge, were used in a volume of 0.3 ml per tube. The tubes were then placed in the incubator for one hour, after which time the medium containing the stool emulsion was drained and replaced with maintenance medium.

Results. In Table I are given the 50% end-point titrations of each of the 3 types of poliomyelitis virus in the 3 different tissue cultures. Results of the first experiment in Table I indicated that the HeLa cells grown in the horse serum medium were less susceptible than were those grown in the human serum or the kidney cells. However, a second experiment indicated an equal susceptibility of all 3 tissue cultures.

In the first of these 2 experiments, on the 5th day following inoculation of the Type I virus at the 10^{-4} dilution when all tubes were showing marked cytopathogenic effect, super-

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† National Institute of Dental Research.

TABLE II. Multiplication of Poliomyelitis Virus in HeLa Cells Grown in Human Serum and in Horse Serum.

Tissue culture	Poliomyelitis virus type			
	I		II	III
HeLa (Human serum)	5.75*	6.5	4.75	7.25
" (Horse ")	5.75	6.5	4.75	6.0

* Titration of 50% end-points in logs/0.1 ml in monkey kidney roller tubes.

nates were harvested from both the horse and the human serum HeLa cell lines. These were titrated in monkey kidney roller tubes. In the second experiment similar harvests were made from the 2 HeLa sublines on the 5th day after virus inoculation from the tubes which had received 10^{-5} dilution of Type I, 10^{-3} dilution of Type II, and 10^{-5} dilution of Type III virus. Again, these harvests were titered in monkey kidney roller tubes. The results of these comparisons of the ability of the 2 sublines of HeLa cells to grow poliomyelitis virus are indicated in Table II, and there is no significant difference between the 2, except perhaps in the one experiment where the human serum HeLa cells apparently gave greater multiplication of the Type III virus.

In an attempt to show the relative efficiency of the 2 sublines of HeLa cells in the isolation of polio virus from human materials, a total of 23 stool emulsions previously shown to contain polio virus, were inoculated into the various tissue cultures. These stool suspensions contained an equal number of materials originally positive in either monkey testicle or kidney cultures for each of the 3 types of poliomyelitis virus. In Table III are shown the results which indicate that the HeLa cells grown in horse serum are just as responsive to polio virus in human stools as those grown in human serum.

In Dr. Earle's laboratory this subline of HeLa cells grown in horse serum medium has

TABLE III. Isolation of Poliomyelitis Viruses from Stools of Patients in HeLa Cells Grown in Human Serum and in Horse Serum.

Tissue culture	Positive stools/total tested	
	Exp. 1	Exp. 2
HeLa (Human serum)	6/10	8/13
" (Horse ")	4/10	10/13
Monkey kidney	—	10/13

been adapted to mass growth in a suspended cell shaker flask type of culture (1,5,6). Because of certain advantages for basic studies on interrelationships between virus and cell of such a culture containing large numbers of viable HeLa cells, multiplying while suspended in the culture medium, 2 experiments were carried out to determine polio virus multiplication in these suspended cells. The suspended cells were grown in Dr. Earle's laboratory. Horse serum adapted HeLa cells grown in stationary flasks were used as the inoculum for the shaker flasks. One flask contained over 400,000,000 cells (approximately 1.6 g wet weight) at the time of inoculation, and the second contained 1,000,000,000 cells (approximately 4 g wet weight).

The total volume of each culture was centrifuged and the sedimented cells were resuspended in shaker flasks in 150 ml of Eagle's maintenance medium containing 4% horse serum. The first flask was inoculated with 3 ml of the 10^{-1} dilution of our standard Mahoney Type I virus, which has a titer of $10^{-7.5}$ per ml. A sample was removed immediately after mixing and again after 24 hours incubation. During incubation the flask was rotated at the rate of 9060 revolutions per hour and was aerated by flushing 4 times with 4 liters of 5% CO_2 in air. The second flask was inoculated with 0.1 ml of the 10^{-3} dilution of the same virus. This flask was rotated at 12,000 r.p.h. and aerated and sampled like the first. Assay of the samples from the first flask carried out in monkey kidney roller tubes showed an increase of virus titer from 10^{-5} per ml at zero hour to greater than $10^{-7.5}$ in 24 hours. Samples from the second flask titered at 10^{-2} at zero hour and $10^{-6.5}$ in 24 hours.

Discussion and Conclusions. Human serum is not readily obtainable in large volumes from a uniform source for use in tissue culture. Furthermore, the possible presence of specific antibodies introduces further difficulties in the use of cells grown in human serum media when working with certain human viruses. The adaptation of HeLa cells to growth in horse serum media eliminates these difficulties.

It would appear from these experiments

that HeLa cells adapted to growth in the presence of horse serum rather than in media containing human serum are quite susceptible to the cytopathogenic effect of all three types of poliomyelitis virus, and are as efficient in producing virus as the HeLa cells grown in the human serum. They likewise seem to be as efficient in their ability to demonstrate the presence of poliomyelitis virus in human stool suspensions. Preliminary evidence indicates that this subline of HeLa cell grown in mass suspended cultures is also capable of supporting the growth of poliomyelitis virus.

1. Perry, V. P., Evans, V. J., and Earle, W. R., *Science*, 1955, v121, 805.
2. Youngner, J. S., *PROC. SOC. EXP. BIOL. AND MED.*, 1954, v85, 202.
3. Eagle, H., *ibid.*, 1955, v89, 96.
4. Black, F. L., and Melnick, J. L., *J. Immunol.*, 1955, v74, 236.
5. Earle, W. R., Bryant, J. C., and Schilling, E. L., *Ann. N. Y. Acad. Sci.*, 1954, v58, 1000.
6. Earle, W. R., Bryant, J. C., Schilling, E. L., and Evans, V. J., *Ann. N. Y. Acad. Sci.*, 1955, in press.

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Glycemic Effects of Chlorpromazine in the Mouse, Hamster and Rat. (21947)

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Various pharmacological effects of chlorpromazine (CPZ) have been reported in the literature among which are depression of body temperature, potentiation of morphine and barbiturates, anti-epinephrine as well as anti-acetylcholine action, and a depression of cellular activity or narcobiosis(1,2,3). The drug which structurally is 10-(3-dimethylaminopropyl)-2-chlorophenothiazine hydrochloride is similar in structure to promethazine but shows very little anti-histaminic property. In view of the various profound effects on the nervous system and cellular activity, the question arose as to the possible effects on blood sugar levels during the post-injection period when this substance is pharmacologically most active.

Materials and methods. The effect of intraperitoneal injections of chlorpromazine (Thorazine, S.K.F.) on blood sugar level of non-fasted and fasted white male mice of the Rockland (RAP) strain was first studied. Animals were injected with CPZ at 5 mg/kg body weight and blood samples were withdrawn *via* the orbital sinus(4), at intervals of 30 minutes, and at 1, 2, 3 and 5 hours later. Non-injected controls were bled at the same

interval periods and blood sugar determinations similarly made. Since the response to CPZ injection in mice was a substantial wave of hyperglycemia, similar experiments were carried out with the hamster and rat. Also the glycemic effects of chlorpromazine in the alloxan diabetic mouse, and the effects, if any, of this substance on the hypoglycemic action of insulin were observed. Mice were separated into 2 groups, each group consisting of 16 animals. Group I was made diabetic by subjecting it to a 48-hour fasting period followed by injection of alloxan monohydrate, 75 mg/kg *via* the intracardiac route(5). After diabetes had been established, these animals were treated with CPZ and the glycemic effects determined. Group II mice received insulin injections in the following manner: Animals were fasted for 24 hours, injected with CPZ, 5 mg/kg, ip, and 2 hours later were injected subcutaneously with insulin, 1/6 unit per 18 g body weight. A minimum of 16 animals was used in all cases for each type of experiment with mice, hamsters and rats. Blood sugar determinations were made by the Somogyi-Nelson method(6).

Results. Normal male mice receiving CPZ

injections of 5 mg/kg, ip, showed an average progressive increase in blood sugar from 142.2 to 202.3 mg% in 30 minutes which by 3 hours had reached 282.8 mg%. A gradual decline in blood sugar then followed which at 5 hours post-injection time had returned to slightly higher than normal value. Non-treated animals which were bled at the same time intervals as treated ones showed a slight blood sugar elevation at the end of the first hour, but all succeeding blood sugar samples were within the normal range.

The hamster showed a hyperglycemic response to CPZ injections similar to that obtained in the mouse although to a lesser degree. The minimal dose required to produce a significant hyperglycemic response was found to be 10 mg/kg when injected intraperitoneally. Hamsters in both the fasted and non-fasted state showed an elevated blood sugar level following CPZ injection, but the hyperglycemia was more pronounced in those animals which had not been starved. The average 24-hour fasting blood sugar level was found to be 96 mg% and 3 hours after injection of CPZ at 10 mg/kg, ip, the glycemic level was raised to 194 mg%. The non-fasted hamster had a glycemic level of 108 mg% which reached 212 mg% at 3 hours after injection of CPZ.

The rat showed very little if any significant rise in blood sugar following injections of CPZ. Various doses were tried ranging from 5 to 60 mg/kg, the latter being approximately the LD₅₀, and in each case the blood sugar level was not appreciably changed. The average fasting glycemia level in the rat was found to be 89 mg% and the maximal glycemic response produced by any dose level was an elevation to 127 mg%. This occurred at 3½ hours following ip injection of CPZ at 20 mg/kg of rat body weight.

Chlorpromazine intensified the fasting hyperglycemia in alloxan diabetic mice and significantly decreased the survival time of these diabetic animals. The average 24-hour fasting blood sugar in the non-treated diabetic mice was 374 mg% while those treated with CPZ showed an elevation to greater than 600 mg% at 2 hours post-injection time. Blood samples were taken in all diabetic mice on al-

ternate days via cardiac puncture with none of the 16 diabetic mice receiving CPZ at 5 mg/kg surviving more than 3 days after such treatment. In contrast to this, 60% of the non-treated alloxan diabetic animals survived a week of alternate day blood withdrawals. In practically all cases of treated and non-treated animals death occurred during the 24-hour starvation period prior to blood sampling. All blood samples were collected in amounts not exceeding 0.2 ml.

Chlorpromazine was observed to afford some protection against the hypoglycemic convulsions and subsequent death produced in mice by injection of 1/6 unit of insulin per 18 g body weight. Of the 16 mice which received insulin at 2 hours after injection of CPZ, 9 animals exhibited convulsions but no deaths occurred in spite of the fact that no glucose was administered. Of the 16 mice receiving the same dose of insulin but no CPZ, 14 convulsed and 11 of these died.

Discussion. It is of interest to speculate on the means by which chlorpromazine brings about so significant a rise in glycemic levels of the mouse and hamster. First, of course, would be the narcobiotic action of this substance which decreases cellular activity so that metabolic oxidation reactions are depressed. This would in effect decrease the amount of glucose being oxidized within the cell and therefore decrease the need or intake of glucose by the cell from extracellular spaces and the blood. As a consequence a rise in blood sugar would occur. Whether or not the depression of cellular activity is the sole means by which CPZ brings about its hyperglycemic effect is difficult to say at this time. The hypothermic effect of this drug, which might be used as an index of cellular activity, is also produced in the rat and yet this animal shows no appreciable hyperglycemic response. The possibility of the endocrine glands such as the adrenals, hypophysis or pancreas acting directly or indirectly through glycogenolysis or gluconeogenesis in the liver has yet to be explored. The profound species variation between the rat and the mouse in the hyperglycemic response to this drug cannot be fully explained at this time. The basal metabolic rate is appreciably higher in the mouse(7).

and this may be the requisite for demonstrating a hyperglycemic response to CPZ since a depression of cellular metabolism in an animal normally functioning at a high metabolic rate would bring about more pronounced changes in the concentration of substances in the blood which are metabolized than would be expected in an animal with a lower normal rate of metabolic activity. Evidence in support of this concept would be the somewhat intermediate effect of CPZ on the blood sugar of the hamster which has a BMR somewhat intermediate to that of the mouse and rat (8,9).

The protection afforded by CPZ from the hypoglycemic convulsions and death following insulin injection in mice would be expected in view of the hyperglycemic state of these animals prior to insulin administration. Whether or not there is any direct antagonism of the insulin molecule cannot be said without further investigation.

Summary. The glycemic effects of chlorpromazine were studied in the mouse, hamster and rat. A hyperglycemic response was found

in the mouse and hamster with the mouse having the greater response of the two. No significant glycemic change was observed in the rat. An exacerbation of the diabetic state and decrease in survival time was found in alloxan diabetic mice treated with CPZ. A protection from hypoglycemic convulsions and death produced by insulin was afforded to mice which had been injected with CPZ 2 hours previous to the injection of insulin.

1. Decourt, P., *J. Pharm. and Pharmacol.*, 1954, v6, 491.
2. Kopera, J., Armitage, A. K., *Brit. J. Pharmacol.*, 1954, v9, 392.
3. Holzbauer, M., and Vogt, M., *ibid.*, 1954, v9, 402.
4. Stone, S. H., *Science*, 1954, v119, 100.
5. Lazarow, A., *J. Lab. and Clin. Med.*, 1947, v32, 1258.
6. Somogyi, M., *J. Biol. Chem.*, 1952, v194, 19.
7. Morrison, P. R., *J. Cell. and Comp. Physiol.*, 1948, v31, 281.
8. Herrington, L. P., *Am. Inst. Physics*, 1941, Reinhold Publ. Corp., N. Y.
9. Kayser, C., *Ann. Physiol.*, 1939, v15, 1087.

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Induction of Toxigenicity in Non-Toxigenic Strains of *C. diphtheriae* with Bacteriophages Derived from Non-Toxigenic Strains. (21948)

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Freeman(1,2) reported that some bacteriophages isolated from *C. diphtheriae* could induce totally non-toxigenic strains of *C. diphtheriae* to become toxigenic and that these transformed strains were indistinguishable in all respects from toxigenic strains occurring in nature. This observation has been confirmed in this laboratory(3,4) as well as in other laboratories(5,6). In each instance, toxigenicity was induced only by bacteriophages obtained from naturally-occurring toxigenic strains of *C. diphtheriae*.

Because of the epidemiological significance of this transformation, studies were undertaken to determine the relative frequency with

which the change could be produced in non-toxigenic strains isolated from cases and carriers. Of the 52 strains tested so far the change has been induced in only 8 strains, all of which were the mitis or mitis-like type. While Hewett(7) has reported the transformation in gravis-type strains employing phages derived from toxigenic gravis strains, it has not been accomplished in this laboratory. However, definitions of the gravis type differ in different laboratories. During serial passage of one group of cultures with phages from toxigenic strains, complete lysis was obtained with 2 non-toxigenic strains, (A-8888, mitis-like North Dakota, and A-8782, gravis,

Alabama) without any demonstrable change in toxigenicity. It appeared probable that these strains were lysogenic in themselves. Therefore, experiments were planned to determine whether or not the bacteriophages isolated from strains A-8888 and A-8782 might induce toxigenicity in other non-toxicogenic strains. Although the data presented here concerning these studies are not complete, sufficient evidence has accumulated to warrant a report.

Materials and methods. Details of the technics used in these experiments will be published elsewhere. The non-toxicogenic strains used in these experiments were chosen from among those which had become toxicogenic when treated with phages derived from toxicogenic strains. All strains of *C. diphtheriae* from which bacteriophage was derived, as well as those strains in which toxigenicity was induced, were tested first for non-toxicogenicity by subcutaneous inoculation in guinea pigs, by intracutaneous inoculation of rabbits, and by the *in vitro* toxigenicity plate method. None showed any evidence of original toxigenicity by any of the methods. The bacteriophages were obtained from atoxigenic strains A-8888 and A-8782 (later also A-8188) by inoculating heart-infusion-agar plates with 3 to 4-hour heart-infusion-broth cultures of the organisms, incubating overnight, followed by extraction of the phage by freezing the agar over CO₂ ice and thawing at room temperature. The expressed liquid was centrifuged at high speed and the supernatant fluid, after heating at 52-53° for 20 minutes, was used in serial passage.

Results. Serial passage of non-toxicogenic Freeman strains F-324 and F-326, with phages obtained from A-8888 and A-8782, produced almost complete clearing after the third passage. Centrifuged sediments of the lysed broth cultures were streaked on the *in vitro* toxigenicity plate and gave the precipitation lines typical of toxicogenic strains of *C. diphtheriae*. A portion of this same sediment, when streaked on heart-infusion-agar plates and incubated overnight, produced partially lysed colonies which also produced lines of precipitation on the *in vitro* toxigenicity plate. Similar centrifuged sediments when injected

intradermally into a rabbit, produced reactions which were indistinguishable from those produced by typical, toxicogenic strains of *C. diphtheriae*. The same results were obtained with 3 separate phage extracts from strains A-8888 and A-8782.

Attempts to induce toxigenicity in other non-toxicogenic strains of *C. diphtheriae* with these same phages were not successful. Curiously, these strains, which failed to become toxicogenic when treated with phages from A-8888 and A-8782, did become toxicogenic when treated with phages derived from known toxicogenic strains. This observation suggests that phages derived from non-toxicogenic strains may differ from phages derived from toxicogenic strains. However, phages derived from non-toxicogenic strains A-8888 and A-8782 and adapted to atoxigenic strains F-324 and F-326 readily induced toxigenicity in non-toxicogenic strains A-334, A-8613, and A-8630. The number of passages necessary to bring about this change varied both with the strain and the phage. Other strains are being passed with these phages but the results are incomplete as yet. The toxigenicity of these phage-changed strains has been tested on both the *in vitro* toxigenicity plate and intradermally in the rabbit.

In another experiment phage was isolated from non-toxicogenic strain A-8188 by the method previously described. After 44 passages with atoxigenic strains F-324 and F-326 only partial lysis of these strains was observed and toxigenicity could not be demonstrated. The heated supernatants from the last passage were used for treatment of non-toxicogenic strains A-8613 and A-8630. Complete lysis was obtained after the seventh passage and the centrifuged sediments were shown to be toxicogenic on the *in vitro* plate and when injected intradermally into the rabbit.

The observations made during the course of the above experiments seem to suggest that there may be different races of diphtheria bacteriophage or that several factors are requisite for induction of toxigenicity not all of which are necessarily present in any given strain of *C. diphtheriae* or in any one bacteriophage.

Summary. It has been established that certain bacteriophages derived from naturally-

occurring non-toxicogenic strains of *C. diphtheriae* will induce toxigenicity in certain non-toxicogenic strains of *C. diphtheriae* as readily as will phages derived from naturally-occurring toxicogenic strains.

1. Freeman, V. J., *J. Bact.*, 1951, v61, 675.
2. Freeman, V. J., and Morse, I. U., *ibid.*, 1952, v63, 407.

3. Parsons, E. I., and Frobisher, M., Jr., *PROC. SOC. EXP. BIOL. AND MED.*, 1951, v78, 746.
4. ———, *Am. J. Pub. Health*, 1953, v43, 269.
5. Hewitt, L. F., *Lancet*, 1952, v2, 272.
6. Groman, N. B., *Science*, 1953, v117, 297.
7. Hewitt, L. F., *J. Gen. Microbiology*, 1954, v11, 272.

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Tissue Conversion of Thyroxine to Triiodothyronine.* (21949)

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The isolation of a new thyroxine-like compound from the thyroid and plasma by Gross and LeBlond(1,2) and the subsequent identification of this substance as 3,5,3'-triiodothyronine by Gross and Pitt-Rivers(3,4), and Roche, Lissitsky and Michel(5) have stimulated a great deal of investigative work. Triiodothyronine has been shown to be about 5 times as potent as thyroxine in preventing thiouracil-induced goiter in rats(6,7,8) and to produce a much more rapid elevation in the oxygen consumption of hypothyroid animals and man than does thyroxine(8-11). These findings, along with the earlier reports by Gross and LeBlond(1) of isolation of the I¹³¹-triiodothyronine from tissues and plasma of thyroidectomized animals given I¹³¹-labeled thyroxine, led Gross and Pitt-Rivers(6) to postulate that thyroxine is enzymatically deiodinated to triiodothyronine in the tissues and that triiodothyronine is the active form of the hormone at the tissue level. Although Albright, Larson and Trust(12) reported the conversion of thyroxine to triiodothyronine by kidney slices and MacLagan and Sprott(13) made similar observations using liver homogenates, Roche, Michel, Michel and Lissitzky (14) have been unable to demonstrate the deiodination of thyroxine to triiodothyronine *in vitro*. More recently Roche and Michel(15), on the basis of *in vivo* work with thyroidecto-

mized animals, found that the thyroid is the sole important source of plasma triiodothyronine and that there was no triiodothyronine or its derivatives in the bile or urine of such animals after the injection of labeled thyroxine. Kalant, Lee, and Sellers(16) reported that triiodothyronine was the major radioactive compound found in the skeletal muscle of propylthiouracil-treated rats given I¹³¹-labeled thyroxine but Taurog(17) stated that his group did not find consistent conversion of thyroxine to triiodothyronine in liver, kidney, spleen or diaphragm.

With these conflicting observations in mind, the following experiments were devised in an attempt to demonstrate the *in vivo* conversion of thyroxine to triiodothyronine by the rat.

Methods. (a) Thirty-three male Sprague-Dawley rats, weighing 250 to 300 g and maintained at 80°F and on standard Purina Laboratory Chow were given 10 μ c (0.5 to 0.7 μ g) of I¹³¹-labeled thyroxine† intravenously. (The stock solution of thyroxine consisted of this hormone in 50% propylene glycol in water: aliquots were diluted with 0.9% saline before administration.) At intervals of 30 minutes, one, 3, 6, 12, and 24 hours after injection, liver, kidney, and skeletal muscle were removed. Nine rats were studied at the 30-minute point, 3 at one hour, 7 at 3 hours, 3 at 6 hours, 4 at 12 hours and 7 at 24 hours.

* This work was supported by a grant from the U.S.P.H.S.

† Obtained from Abbott Laboratories, Oak Ridge, Tenn.

These tissues were homogenized in NH_4Cl buffer (pH 8.4), boiled for 30 minutes, digested with crude trypsin for 20 hours and extracted 3 times with *n*-butanol. The butanol extract was then evaporated to dryness and the residue taken up in 2 to 3 ml of 0.5 N NaOH. Identical experiments were performed either using purified trypsin instead of crude trypsin or omitting trypsin digestion entirely with essentially the same results except that digestion with either purified or crude trypsin produced a slightly higher yield of butanol-extractable material. The material taken up in NaOH was then run through kieselguhr chromatographic columns according to the method of Gross and Pitt-Rivers(4)[†] and Braasch, Flock and Albert(18). Butanol containing 20% chloroform equilibrated with 0.5N NaOH was run through the column until thyroxine, triiodothyronine and iodide were eluted. The solvent system was then changed to 10% isopropyl alcohol in butanol for the purpose of recovering di- and monoiodotyrosine. The effluent was collected with a fraction collector and the radioactivity of each fraction was assayed with a scintillation detector. The results were expressed graphically, plotting counts per minute in the fraction versus fraction number. The relative amounts of all radioactive components recovered was determined roughly by comparing the areas under the peaks obtained. (b) An experiment using 16 rats was performed identical to that described above except that 10 μc (0.5 to 0.7 μg) of I^{131} -labeled triiodothyronine was injected into the animals. The animals were sacrificed 1, 3, 6 and 12 hours after the injection of triiodothyronine. Four animals comprised each group. (c) An experiment similar to that described under (a) was carried out in 16 animals 2 weeks after thyroidectomy. In this case 10 μc of thyroxine which had been re-purified by passage of the stock solution through the kieselguhr column was injected intravenously and the animals sacrificed 30 minutes after injection. Specimens of liver, kidney, and muscle were homogenized and

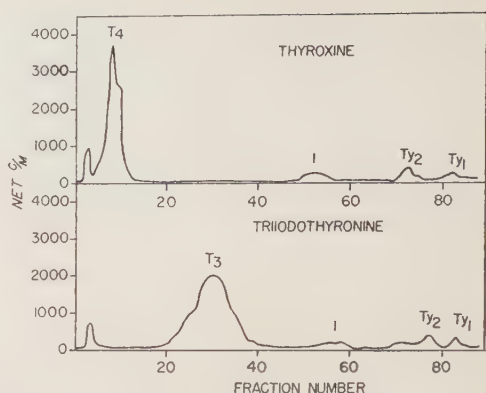


FIG. 1. Representative graphs of kieselguhr chromatographic analysis of liver, kidney and muscle at various time intervals after injection of (a) I^{131} -labeled thyroxine and (b) I^{131} -labeled triiodothyronine. T_4 indicates thyroxine; T_3 , triiodothyronine; I, iodide; Ty_1 , monoiodotyrosine, and Ty_2 , diiodotyrosine.

treated as described above. Other specimens of liver and kidney were homogenized in sucrose solution at 4°C and separated, by means of differential ultracentrifugation(19), into nuclear, mitochondrial, microsomal, and residual protein fractions. The fractions thus obtained were treated as described in the initial part of the experiment and the extracts were subjected to kieselguhr chromatographic analysis.

Recovery studies with either labeled thyroxine or labeled triiodothyronine revealed that 98 to 101% of the radioactivity placed on the column was recovered in the effluent.

Results. Normal rats, whole tissues. Fig. 1 is representative of the results obtained in liver, kidney or muscle from intact normal rats following the administration of labeled thyroxine and triiodothyronine. The chromatographs were essentially alike at all time intervals studied. When thyroxine was administered the main compound recovered corresponded to thyroxine on chromatographic analysis, and in most instances no triiodothyronine was recovered. However, in 20% of the total number of chromatographic analyses, small triiodothyronine peaks were observed following the administration of thyroxine. Fig. 2 characterizes the findings in these instances. The triiodothyronine peak was present at all of the various time intervals following injection, including the 30-minute point

[†] The authors wish to express their gratitude to Dr. Jack Gross for his invaluable help and advice in the use of the kieselguhr chromatographic technic.

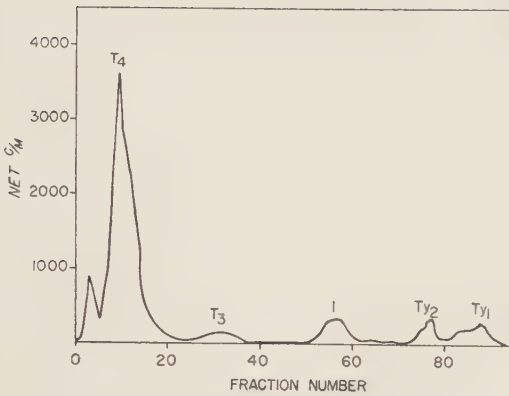


FIG. 2. Plot of radioactivity recovered from kieselguhr columns representative of instances (20% of total assays) where triiodothyronine was recovered from liver, kidney and muscle after injection of I^{131} -labeled thyroxine.

and, although recovered occasionally from all 3 tissues, it was found more frequently in liver and kidney than in muscle. When the approximate relative amount of radioactivity present in each of the chromatographic peaks was determined by measuring the area under each peak, the radioactivity corresponding to triiodothyronine never exceeded 1.5% of the total.

When triiodothyronine was administered, triiodothyronine was the main product of the column separation and no thyroxine was observed at any time. *Thyroidectomized animals:* In the 16 thyroidectomized animals injected with thyroxine and sacrificed 30 minutes later, essentially the same results were observed as in the normal animals. In approximately 20% of all analyses done on liver, kidney and muscle in these animals, small triiodothyronine peaks were detected, the traces of this substance occurring apparently at random in all 3 tissues. In no case did the triiodothyronine peak comprise more than 1.5% of the total radioactivity recovered.

Subcellular fractionations. Fig. 3 is illustrative of the observations made after thyroxine injection into thyroidectomized rats when subcellular fractions (microsomes, mitochondria, nuclei and residual protein) of liver and kidney were subjected to chromatographic separation. Similar findings occurred in the fractions of both liver and kidney. In

cases where traces of triiodothyronine were found in the whole homogenates of these tissues, relatively greater amounts of this compound were isolated from some of the subcellular fractions. The greatest concentrations of triiodothyronine appeared in the microsomes and residual fraction of both liver and kidney, the amount of triiodothyronine running as high as 10.5% of the total radioactivity in the case of soluble protein fraction of the kidney. It was of great interest that even in some cases where no triiodothyronine was found in the chromatographic separation of whole homogenates, significant amounts of triiodothyronine were found in the subcellular fractions.

Discussion. By the method of kieselguhr chromatographic separation, we were able to recover triiodothyronine from whole rat tissues following the administration of thyroxine. Triiodothyronine was recovered from liver, kidney and muscle in about 20% of the chromatographic analyses made. When it was found, it was present in only small amounts, never exceeding 1.5% of the total radioactivity recovered. These findings are somewhat at variance with those of Kalant *et al.* with respect to the amounts of triiodothyronine recovered. If thyroxine is actually metabolized to triiodothyronine, and if the appear-

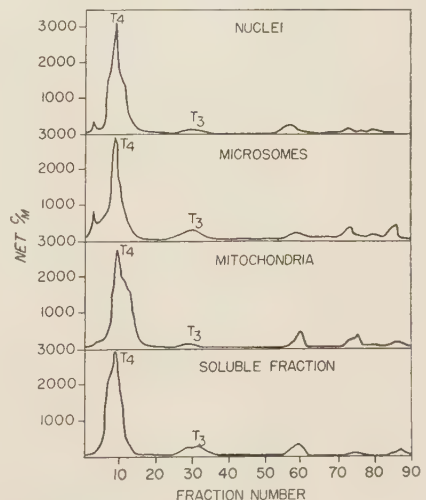
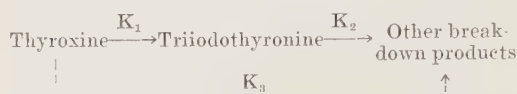


FIG. 3. Radioactivity in effluent from kieselguhr columns representative of findings in subcellular fractions of liver and kidney following injection of I^{131} -labeled thyroxine.

ance of triiodothyronine is not an artifact produced by the conditions of this experiment, it may be that only a relatively small percentage of thyroxine is converted to triiodothyronine. However, since triiodothyronine, once formed, may be further degraded very rapidly, and hence not be recovered from tissue as such, it is not possible to estimate accurately the amount converted.

If such a conversion does actually occur, the mechanism might be simply expressed as follows:



It is probable that rate constant K_2 is at least 3 or 4 times K_1 . If this is the case one would expect to find relatively small amounts of triiodothyronine in the tissues involved at any one time, no matter what percentage of thyroxine is degraded via this pathway.

It may be argued that only small amounts of triiodothyronine were recovered and this only relatively infrequently because of poor recovery during the experimental procedure. This point may be answered in part by pointing out that when stock triiodothyronine was subjected to the experimental chromatographic conditions, more than 95% was recovered as triiodothyronine. However, under our experimental conditions we have consistently observed a "front" peak of radioactivity which is recovered from the kieselguhr columns before the thyroxine peak appears. (Figs. 1, 2, 3). Efforts to identify the radioactive components of this peak by paper chromatographic techniques have so far been unrewarding and it is entirely possible that small amounts of triiodothyronine may be included in this material. This might explain the appearance of triiodothyronine, following the administration of thyroxine, at some times and not at others.

Similarly, it may be argued that the appearance of triiodothyronine after administration of thyroxine was due to deiodination produced by the conditions of the experiment rather than to tissue activity. However, when thyroxine stock solution was subjected to the ex-

perimental conditions no triiodothyronine was recovered.

The appearance of triiodothyronine in the subcellular fractions in greater concentrations than in the whole homogenate is as yet unexplained. The largest amounts appeared in the microsomes and residual fraction in both liver and kidney, probably due to concentration in these fractions. Concentration alone, however, does not explain the consistent appearance of triiodothyronine in the subcellular fractions in contrast to its irregular appearance in the whole homogenate, since the concentration in all cell fractions was higher than that observed in the whole homogenate. It was noted during the course of our experiments that the "front" peak was consistently smaller in the instances where the subcellular fractions were subjected to kieselguhr chromatographic separation than was the case with the whole homogenate, probably due to minor alterations in the experimental conditions. If this "front" contains appreciable amounts of triiodothyronine this difference might explain the more constant appearance of this substance in the analyses where the "front" was smaller.

Our observations have indicated that a small amount of thyroxine may be converted to triiodothyronine in rat tissues. However, it seems less likely that the main mode of action of thyroxine involves this step. In view of the recent works of Thibault and Pitt-Rivers(20), the possibility seems stronger that both substances exert their action only after further alteration, perhaps involving conversion to the acetic acid or other des-amino analogues.

Summary. (1) I^{131} -labeled thyroxine was administered intravenously to normal and thyroidectomized rats and the animals sacrificed at varying time intervals thereafter. Specimens of liver, kidney and muscle were homogenized, extracted with butanol and the extracts were subjected to kieselguhr chromatographic analysis. Small amounts of triiodothyronine were recovered from all 3 tissues in about 20% of the analyses carried out. (2) When triiodothyronine was administered and the tissues subjected to the same conditions, no thyroxine was recovered. (3) When

the tissues of rats given thyroxine were homogenized and subjected to fractionation into their subcellular components, triiodothyronine was more readily recovered than was the case where the whole homogenate was used. A possible explanation for this is discussed.

1. Gross, J., and LeBlond, C. P., *Endocrinology*, 1951, v48, 714.
2. ———, *Proc. Soc. Exp. Biol. and Med.*, 1951, v76, 688.
3. Gross, J., and Pitt-Rivers, R., *Lancet*, 1952, v262, 439.
4. ———, *Biochem. J.*, 1953, v53, 645.
5. Roche, J., Lissitzky, S., and Michel, R., *Compt. rend. Acad. Sc.*, 1952, v234, 1228.
6. Gross, J., and Pitt-Rivers, R., *Lancet*, 1952, v262, 593.
7. ———, *Biochem. J.*, 1953, v53, 652.
8. Tomish, E. B., and Woollett, E. A., *Lancet*, 1953, v264, 726.
9. MacLagan, N. F., Sprott, W. E., and Wilkinson, J. H., *ibid.*, 1952, v263, 915.

10. Heming, A. E., and Holtkamp, D. E., *Fed. Proc.*, 1953, v12, 330.
11. Gross, J., Pitt-Rivers, R., and Trotter, W. R., *Lancet*, 1952, v262, 1044.
12. Albright, E. C., Larson, F. C., and Trust, R. H., *Proc. Soc. Exp. Biol. and Med.*, 1954, v86, 137.
13. MacLagan, N. F., and Sprott, W. E., *Lancet*, 1954, v267, 368.
14. Roche, J., Michel, R., Michel, D., and Lissitzky, S., *Compt. rend. Soc. Biol.*, 1951, v145, 228.
15. Roche, J., and Michel, R., *Acta Endocrinologica*, 1954, v17, 385.
16. Kalant, H., Lee, R. B., and Sellers, E. A., *Endocrinology*, 1955, v56, 127.
17. Taurog, A., *The Thyroid*, Brookhaven Symposia in Biology, 1954, No. 7, 88.
18. Braasch, J. W., Flock, E. V., and Albert, A., *Endocrinology*, 1954, v55, 768.
19. Lee, N. D., and Williams, R. H., *ibid.*, 1954, v54, 5.
20. Thibault, O., and Pitt-Rivers, R., *Lancet*, 1955, v268, 285.

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Intracellular Potassium Concentration in Normal and Diabetic Rat Liver.* (1950)

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It has previously been shown that marked abnormalities of carbohydrate metabolism occur in liver slices from normal animals incubated under conditions leading to lowered intracellular potassium concentration(1). Thus, potassium depletion leads to decreased glycogen deposition from glucose and to decreased overall glucose utilization, while gluconeogenesis from pyruvate and other precursors is markedly increased. These metabolic abnormalities are qualitatively similar to those found in liver slices from diabetic rats even in the presence of normal intracellular

potassium concentration(2,3). Furthermore, a number of observations have been made, both clinically and in experimental animals, suggesting that potassium depletion may lead to disturbances of carbohydrate metabolism qualitatively similar to those of diabetes mellitus(4,5). It seemed of interest, therefore, to determine whether lowered intracellular potassium concentration occurred in diabetic liver *in vivo*. Accordingly, electrolyte, water and glycogen analyses were made on diabetic and normal rat livers.

Material and methods. Male rats weighing 250 g were made diabetic by the intravenous injection of alloxan (40 mg per kilo). Three to 4 weeks later these animals, as well as normal controls, were fasted for 8 hours (water *ad lib.*), then sacrificed for blood and liver analysis. The animals were sacrificed under nembutal anesthesia by severing the carotid

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TABLE I. Blood Analysis on Normal and Diabetic Rats.

Group	Blood sugar, mg/100 ml blood		Na	K
	Mean Range		mEq/L serum	
Normal (11)	99	78-117	149.1 \pm 6.4*	4.74 \pm 1.5*
Diabetic (11)	292	121-470	144.7 \pm 3.8	4.25 \pm 1.8

* Mean values \pm stand. error of mean.

arteries. The livers were removed, blotted free of blood, and 2 aliquots were taken. One was for glycogen analysis(6) and the other was used to determine water, sodium and potassium. This second aliquot was dried, defatted and extracted with 0.75 N HNO_3 as described by Lowry and Hastings(7). Sodium and potassium were determined by flame photometry. Whole blood was also analyzed for glucose(8) and blood serum for sodium and potassium.

Results. Table I shows the blood sugar and serum sodium and potassium values for 11 normal and 11 diabetic animals. The serum sodium and potassium concentrations were slightly lower in the diabetic than in the normal group. These data were used in calculating the intracellular potassium concentrations.

In Table II are recorded the analytical data obtained on the normal and diabetic livers, together with calculated values for extracellular water (H_2O)_e, intracellular water (H_2O)_i and intracellular potassium concentration $[\text{K}]_i$. From the analytical data it may be seen that no statistically significant difference was ob-

served in the concentrations of water, potassium or sodium between the normal and diabetic livers.

Using these data, and the assumption that the sodium of liver is entirely extracellular, the extracellular water (H_2O)_e and intracellular water (H_2O)_i, in g per kilo of fat free tissue, were calculated according to methods previously described(7). No difference in either (H_2O)_e or (H_2O)_i was found between the livers of the normal and diabetic groups.

After making allowance for the amount of potassium in the extracellular water of the tissues, the concentration of potassium in the intracellular water $[\text{K}]_i$ was calculated. These values proved to be 187 mEq per kilo of intracellular water for the normal livers and 184 mEq per kilo of water for the diabetic livers. The difference is without significance.

Discussion. Since abnormalities in carbohydrate metabolism similar to those encountered in diabetic liver have been observed in normal liver where intracellular potassium concentration is experimentally reduced below normal, we have felt it important to determine whether the intracellular potassium concentration of liver is or is not reduced below normal in diabetes. From the data presented above it would appear that the intracellular potassium concentration is quite normal in diabetic liver. It would seem, therefore, that the metabolic abnormalities which have been described in hepatic tissue for diabetic animals are not attributable to a reduction in potassium concentration but rather it appears more likely that potassium depletion and insulin de-

TABLE II. Analytical Data on Normal and Diabetic Rat Livers. Mean values are expressed per kg of fat-free tissue (F.F.T.)—standard error of the mean.

Group	H_2O , g	K, mEq	Na, mEq	Glycogen, g
Normal (11)	715 \pm 1.5	96.2 \pm .6	31.4 \pm 2.1	7.0 \pm 1.2
Diabetic (11)	723 \pm 2.8	95.5 \pm 1.0	30.5 \pm 1.2	19.4 \pm 2.2
Derived data				
	(H_2O) _e *, g/kg F.F.T.	(H_2O) _i †, g/kg F.F.T.	$[\text{K}]_i$, mEq/kg (H_2O)	$[\text{K}]_i$, mEq/100 g F.F. solids
Normal (11)	198 \pm 15	517 \pm 15	187 \pm 8	33.8 \pm .2
Diabetic (11)	198 \pm 7	515 \pm 7	184 \pm 4	33.3 \pm .2

* (H_2O)_e—g of extracellular H_2O per kg of F.F.T., calculated on the assumption that liver Na is extracellular.

† (H_2O)_i—g of intracellular H_2O per kg of F.F.T., calculated from total tissue H_2O and (H_2O)_e.

iciency affect intracellular metabolism similarly but independently.

Summary. The intracellular potassium concentration was found to be normal in diabetic rat liver.

1. Hastings, A. B., Teng, C. T., Nesbett, F. B., and Sinex, F. M., *J. Biol. Chem.*, 1952, v194, 69.
2. Renold, A. E., Teng, C. T., Nesbett, F. B., and Hastings, A. B., *ibid.*, 1953, v204, 533.
3. Hastings, A. B., Renold, A. E., and Teng, C. T., *Trans. Assn. Am. Phys.*, 1953, v66, 129.

4. Gardner, L. I., Talbot, N. B., Cook, C. D., Bermenand, H., and Uribe, R., *J. Lab. and Clin. Med.*, 1950, v35, 592.

5. Eliel, L. P., Pearson, O. H., and White, F. C., *J. Clin. Invest.*, 1952, v31, 419.

6. Good, C. A., Kramer, H., and Somogyi, M., *J. Biol. Chem.*, 1933, v100, 485.

7. Lowry, O. H., and Hastings, A. B., *ibid.*, 1942, v143, 257.

8. Somogyi, M., *ibid.*, 1945, v160, 61.

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Renin and Antirenin in Treatment of Long Term Experimental Renal Hypertension in the Dog.* (21951)

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A number of reports have suggested that the pathogenesis of experimental renal hypertension changes with time. Thus it has been suggested that in acute experimental renal hypertension the pathogenesis is renal and in chronic experimental renal hypertension the pathogenesis is extrarenal or (according to one group of investigators) neurogenic. There have been no reported studies on the pathogenesis of long term experimental renal hypertension (*i.e.*, hypertension of 4 or more years duration) in the dog. Thus Ogden and colleagues(1,2) showed that certain sympatholytic and general anesthetic drugs decreased the blood pressure of chronic renal hypertensive rats but not of acute renal hypertensive rats. They also showed that the removal of a unilaterally constricted kidney in the rat caused a decrease in blood pressure in acute but not in chronic renal hypertensive rats. On the basis of these findings, Ogden suggested that the pathogenesis of experimental renal hypertension changes with time from a renal pressor mechanism to a neurogenic mechanism. Likewise, several groups of British investigators have reported that complete nephrec-

tomy in the acute renal hypertensive rat produces a decrease in blood pressure, whereas there is no decrease during survival in the chronic hypertensive rat(3,4). These results are similar to those of earlier workers who reported that total nephrectomy in renal hypertension of one week's duration in the rabbit results in normotension but after 2 to 4 months of hypertension, total nephrectomy results in persistence of the hypertension during survival(5,6). Floyer and others have interpreted these findings as demonstrating an extrarenal mechanism for the pathogenesis of chronic experimental renal hypertension. Moss and Wakerlin(7) and Page(8) however, found no significant difference in the effects of sympatholytic and general anesthetic drugs in acute and chronic experimental renal hypertension in dogs. Moss and Wakerlin considered acute experimental renal hypertension in dogs to last 6 months following renal artery constriction and most of their acute hypertensive dogs were 3 to 6 months hypertensive. Since then, Haynes and Dexter(9) showed that an increase in plasma renin following renal artery constriction in the dog persists for some weeks and Shorr and colleagues(10) showed that increased plasma VEM is balanced by VDM 8 to 12 weeks following renal

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artery constriction in dogs. These observations suggest that the dividing line between acute and chronic experimental renal hypertension in dogs may be 3 rather than 6 months. In keeping with this, Ohler and Wakerlin(11) found that dibenzyline produced no decrease in the blood pressure of 7 renal hypertensive dogs within 3 months following renal artery constriction whereas a decrease occurred in dogs which were hypertensive for 6 months or more. However, the decrease was not to the normotensive level and certainly not to the hypotensive level produced by dibenzyline in normotensive dogs. Recently, Ogden and colleagues(12) reported that pentobarbital decreased the blood pressure of dogs with renal hypertensions of more than one year's duration but not to normotension. These findings suggest that vasomotor tonus is in abeyance during acute renal hypertension but returns to at least a normal level during chronic renal hypertension in the dog.

To study the pathogenesis of long term experimental renal hypertension in dogs, we observed the effect of semipurified hog renin therapy and of passive immunization with antirenin to hog renin in dogs with renal hypertensions of 50 to 121 months duration. Two years ago, our research group showed that a course of semipurified hog renin from renal cortex protected against the development of experimental renal hypertension in dogs and was effective in the treatment of chronic experimental renal hypertension of 8 to 37 months duration in dogs, whereas numerous control tissue extracts were without effect(13). Since the effectiveness of prophylaxis and treatment was well-correlated with the serum antirenin titre, the findings were considered good evidence for the hypothesis that renin (or a closely related renal protein) plays the principal pathogenetic role in experimental renal hypertension in the dog for at least the first 3 years. A pathogenetic role for renin is also suggested by the antihypertensive effect of homologous antirenin to hog renin passively transferred to renal hypertensive dogs(14).

Methods. Ten dogs with renal hypertensions of 50 to 121 months duration were treated for 6 to 12 months with one or two

courses of daily intramuscular injections of semipurified hog renin from cortex in doses varying from 2.5 to 20.0 D. U. (Goldblatt) per kg. The semipurified hog renin was prepared by a modification of the method of Marshall and Wakerlin(15) or the method of Haas and Goldblatt(16). The purity of the renin varied from 16-175 D. U. per mg N, but most of it was 75-175 D. U. per mg N. Antirenin titres on the serum were determined monthly by a method already described(13). The time between courses of hog renin therapy in the 6 dogs receiving a second course of hog renin was at least 6 months, during which the blood pressure reached its pretreatment hypertensive level and the antirenin titre fell to 0. Male and female dogs were made hypertensive by a standardized modification of the Goldblatt technique of renal artery constriction already reported(13). Mean blood pressure readings were obtained by femoral artery puncture one or 2 times per week, with occasional comparison by the strain gauge technic. Studies of blood urea nitrogen, urinalyses, and determinations of body weight and clinical condition were made at monthly intervals or more often when indicated.

For the passive transfer study, 3 of the dogs with renal hypertensions of 93 to 108 months duration were given high titre homologous antiserum containing antirenin to hog renin. Each of these dogs had previously been treated with a course of semipurified hog renin with a sufficient lapse of time (6 months or more) to permit a decrease in the antirenin titre to 0 and a return of the blood pressure to the pretreatment hypertensive level for 2 to 3 months. Blood pressure was measured every other day for one week prior to antirenin administration and the antirenin titre was determined to be 0 A. U. per cc of serum. Antirenin in total amounts varying from 947 A. U. to 3,560 A. U. per kg was administered subcutaneously in 4 divided doses over a 2-day period. Blood pressures and serum antirenin titres were determined on the 3rd, 5th, and 7th days following the first day of antirenin administration and subsequently at weekly or semi-weekly intervals. Urinalyses were made prior and subsequent to the administration of antirenin and the clinical condition of the dogs

TABLE I. Antihypertensive Effect of Semipurified Hog Renin from Cortex in Experimental Renal Hypertension in 10 Dogs with Hypertensions of 50 to 121 Months Duration.

Dog No.	Sex	Max anti- renin titre (A.U./cc)	Duration of hyper- tension	Blood pressure in mm Hg		Max re- sponse to treatment	Effect on hypertension, %
				Normotension	Hypertension		
1	♀	37	50	125 ± 7.0*	183 ± 3.7*	151 ± 5.8*	-55
2	♀	29	51	122 ± 7.8	162 ± 5.4	153 ± 7.2	-23
3	♂	32	51	123 ± 5.0	167 ± 7.5	129 ± 8.0	-86
4	♂	0	51	115 ± 5.4	201 ± 6.9	219 ± 10.5	+21†
5	♀	50	56	127 ± 6.8	158 ± 3.8	142 ± 4.8	-50
6	♂	24	60	120 ± 6.8	185 ± 4.0	140 ± 10.5	-69
7	♀	18	66	117 ± 5.2	185 ± 10.5	138 ± 8.4	-68
8	♂	20	67	127 ± 9.1	172 ± 5.5	147 ± 6.6	-55
2	♀	68	69	122 ± 7.8	172 ± 4.8	148 ± 4.0	-48
9	♂	22	71	117 ± 4.6	164 ± 9.4	145 ± 7.6	-47
7	♀	21	81	117 ± 5.2	166 ± 6.7	140 ± 3.9	-53
9	♂	85	82	117 ± 4.6	170 ± 11.0	142 ± 6.6	-53
8	♂	16	87	127 ± 9.1	166 ± 5.8	150 ± 6.6	-41
3	♂	40	98	123 ± 5.0	171 ± 7.0	147 ± 9.1	-50
10	♂	0	99	129 ± 9.0	160 ± 2.7	183 ± 7.2	+74†
10	♂	11	121	129 ± 9.0	166 ± 3.2	146 ± 5.9	-54

* Avg ± stand. dev.

† Maintained chronic pressor effect in the absence of antirenin.
The P value (t test) was <.01 in all experiments.

was observed daily. Control experiments with normal serum were conducted.

Results. Table I summarizes results with hog renin injections. The normotensive and hypertensive blood pressure levels are each based upon a minimum of 12 to 18 readings over a period of 2 to 3 months. The maximal response to treatment level is based on a minimum of 6 to 12 readings over a period of one to 2 months. All of the dogs showed a significant antihypertensive effect ranging from 23 to 86% reduction in their hypertensions, except the 2 animals which did not develop antirenin. None of the dogs showed adverse effects from treatment. Appetite, body weight, and general clinical condition were well maintained. Urinalyses and blood urea nitrogen determinations consistently gave nor-

mal findings.

The maximal antihypertensive effect for the first course of injections occurred during the third to the sixth months of treatment as the antirenin titre approached its maximum and was maintained until treatment was discontinued. The maximal antihypertensive effect for the second course was observed during the second to the fourth months of treatment, correlating with the more rapid anamnestic development of antirenin. Following the end of treatment, the blood pressure gradually returned to its pretreatment level over a period of 2 to 6 months as the antirenin titre fell toward 0. Fig. 1 shows a typical antihypertensive effect from a course of semipurified hog renin in a long term renal hypertensive dog. The 2 dogs which did not develop anti-

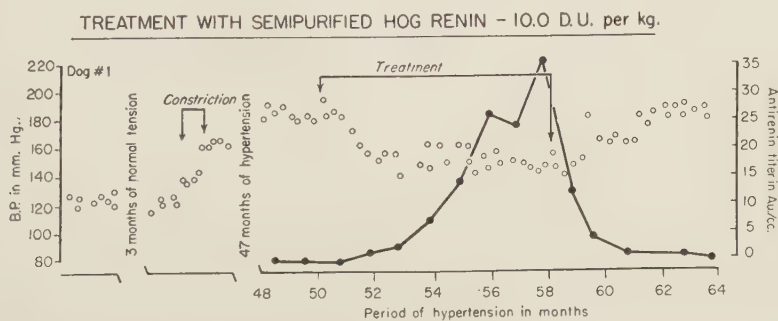


FIG. 1.

TABLE II. Antihypertensive Effect of Homologous Antirenin to Hog Renin Passively Transferred to Dogs with Long Term Experimental Renal Hypertension.

Dog No. (see Table I)	Sex	Total anti- renin dose (A.U./kg)	Max anti- renin titre	Duration of hyper- tension	Blood pressure in mm Hg—			Effect on hyperten- sion, %
					Normo- tension	Hyper- tension	Max re- sponse to antirenin	
7	♀	3560	9	93	117 ± 5.2*	169 ± 11.6*	128	- 79
7	♀	947	5	97	117 ± 5.2	177 ± 9.0	102	-100
7	♀	0	0	99	117 ± 5.2	166 ± 8.7	164 ± 7.0	- 4*
9	♂	1353	4	96	117 ± 4.6	164 ± 4.0	133	- 66
10	♂	1700	15	108	129 ± 9.0	164 ± 2.5	114	-100
10	♂	0	0	114	129 ± 9.0	166 ± 5.2	166 ± 3.0	0*

* Normal serum controls.

renin not only did not show an antihypertensive effect but showed a maintained chronic pressor effect which has been discussed elsewhere(17).

Table II summarizes results with the passive transfer of antirenin. The 4 passive transfer experiments with antirenin produced 66 to 100% reductions in the hypertensions of the three long term renal hypertensive dogs compared with antihypertensive effects in the same dogs of 54 to 68% as a result of 5 courses of hog renin. The maximal antihypertensive effect was obtained on the third or fifth day of the passive transfer experiment, usually simultaneously with the maximum antirenin titre. The blood pressure then gradually returned to the previous hypertensive level over a period of two weeks or more as the antirenin titre fell toward 0. Control passive transfer experiments with normal serum in 2 of the 3 dogs were without antihypertensive effect. None of the animals showed adverse changes in clinical condition or abnormalities in urinalyses following antiserum or serum administrations. Fig. 2 illustrates a passive transfer of antirenin and a control experiment in one of the dogs.

Discussion. The results with hog renin and

with the passive transfer of antirenin support the hypothesis that renin (or a closely related renal protein) plays a significant role in experimental renal hypertension of many years duration in the dog. Although our research group has already shown that numerous control tissue extracts are without antihypertensive effect(13), the lack of response in the 2 long term hypertensive dogs failing to develop antirenin on hog renin therapy, further rules out a non-specific foreign protein effect as the cause of the antihypertensive response. The failure of most of the long term hypertensive dogs to show a decrease in blood pressure to normotension whereas most of the dogs with chronic hypertensions of 8 to 37 months showed such a decrease on semipurified hog renin therapy(13), suggests at least three possible explanations: (1) the pathogenesis of experimental renal hypertension in the dog may undergo a gradual change with the passage of years whereby renin plays a decreased role; (2) progressive arteriolosclerosis may develop over a period of years; and (3) compensatory mechanisms opposing the antihypertensive effect of actively produced antirenin may be enhanced in long term as contrasted with chronic experimental renal hypertension in the dog. The decrease in blood pressure to normotension or near normotension produced by the passive transfer of antirenin is opposed to (1) and (2). Likewise an extensive study of the passive transfer of antirenin in renal hypertensive dogs to be published later(18) indicates that the duration of chronic hypertension is without effect on the antihypertensive response to passive immunization with antirenin. Moreover, histological examination of the arterioles of long term hy-

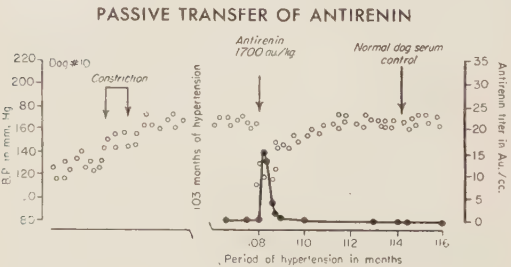


FIG. 2.

pertensive dogs by us confirmed the previous observation of Goldblatt that experimental renal hypertensive dogs show a minimal amount of arteriolosclerosis. This leaves the suggestion that compensatory hypertensive mechanisms come more prominently into play when serum antirenin is gradually increased by semipurified hog renin than when the serum antirenin titre is quickly developed by passive immunization with antirenin. Our observations shed no light on what these compensatory mechanisms may be although increased vasomotor tonus may be involved. Recently our research group reported an increase in renal renin concentration proportional to the antirenin titre in normotensive and renal hypertensive dogs on hog renin therapy but found no difference in this respect among acute, chronic, and long term renal hypertensive dogs(19).

Floyer(20) has suggested that possibly renin may exercise its pathogenetic role in chronic experimental renal hypertension by acting on the kidney perhaps to decrease the production of the blood pressure regulatory or depressor renal hormone (loss of which by complete nephrectomy results in renoprival hypertension)(21). Conceivably this action might evolve gradually during acute experimental renal hypertension and become significant only in chronic experimental renal hypertension. This hypothesis would reconcile the conflicting findings reviewed in the introduction of this report and would be in agreement with our finding that renin still plays a dominant role in the pathogenesis of long term experimental renal hypertension. The decreased antihypertensive response of long term experimental renal hypertension to hog renin injections might be due to failure of the blood pressure regulatory renal hormone to increase to the normal level as the serum antirenin titre is gradually increased by active immunization with hog renin. Certainly this intriguing hypothesis should be investigated.

Summary and conclusions. 1. Courses of intramuscular injections of semipurified hog renin from cortex administered to dogs with experimental renal hypertensions of 4 or more years duration produced a significant reduc-

tion in blood pressure in those dogs which developed antirenin but not to normotension as previously found in dogs with chronic renal hypertension of 6 to 37 months duration. The 2 dogs which did not develop antirenin showed a maintained chronic pressor effect. 2. Passive immunization with homologous antirenin to hog renin produced a reduction in blood pressure to normotension or near normotension in dogs with experimental renal hypertensions of more than 7 years duration. 3. The mechanism of the decreased response to hog renin therapy in long term experimental renal hypertension in the dog is unknown. The results with passive immunization suggest that a lessened pathogenetic role for renin is not involved nor is progressive arteriolosclerosis. Possibly compensatory hypertensive mechanisms are more effective in the face of the gradually increasing antirenin titre of hog renin therapy than of the abruptly produced antirenin titre of passive immunization. 4. The results indicate that renin (or a closely related renal protein) is the principal pathogenetic factor even in experimental renal hypertension of many years duration in the dog.

1. Reed, R. K., Sapirstein, L. A., Southard, F. D., and Ogden, E., *Am. J. Physiol.*, 1944, v41, 707.
2. Ogden, E., Collings, W. D., and Sapirstein, L. A., *Spec. Pub. N. Y. Acad. Sc.*, 1946, v3, 153.
3. Floyer, M. A., *Clin. Sc.*, 1951, v10, 405.
4. ———, Ciba Foundation Symposium on Hypertension, Boston, 1954, p. 155, Little, Brown & Co.
5. Pickering, G. W., *Clin. Sc.*, 1945, v5, 239.
6. Daniel, P. M., Prichard, M. M. L., and Ward-McQuaid, J. N., *ibid.*, 1954, v13, 247.
7. Moss, W. G., and Wakerlin, G. E., *Am. J. Physiol.*, 1950, v161, 435.
8. Page, I. H., *Factors Regulating Blood Pressure*, Trans. First Conf., New York, Josiah Macy, Jr. Found., 1947, p18.
9. Haynes, F. W., and Dexter, L., *Am. J. Physiol.*, 1947, v150, 190.
10. Shorr, E., *Hypertension*, A Symposium, Univ. of Minn. Press, 1950, p86.
11. Ohler, E. A., and Wakerlin, G. E., *Factors Regulating Blood Pressure*, Trans. 5th Conf., New York, Josiah Macy, Jr., Found., 1951, p111.
12. Mandel, M. J., Greene, R. W., Sapirstein, L. A., and Ogden, E., *Am. J. Physiol.*, 1954, v176, 352.
13. Wakerlin, G. E., Bird, R. B., Brennan, V. B., Frank, M. H., Kremen, S., Kuperman, I., and

- Skom, J. H., *J. Lab. and Clin. Med.*, 1953, v41, 708.
14. Kuperman, I., and Wakerlin, G. E., *Fed. Proc.*, 1953, v12, 80.
15. Marshall, J., and Wakerlin, G. E., *ibid.*, 1949, v8, 106.
16. Haas, E., Lamfrom, H., and Goldblatt, H., *Arch. Biochem. and Biophysics*, 1953, v42, 368.
17. Wakerlin, G. E., Kremen, S., Frank, M. H., Schmid, H. E., and Graham, L., *Circulation Res.*, 1954, v2, 201.
18. Wakerlin, G. E., Kuperman, I., Schmid, H. E., and Graham, L., to be published.
19. Schmid, H. E., and Wakerlin, G. E., *Fed. Proc.*, 1955, v14, 132.
20. Floyer, M. A., personal communication, 1955.
21. Grollman, A., Muirhead, E. E., and Vanatta, J., *Am. J. Physiol.*, 1949, v157, 21.

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Technic for Preparation of Glycerol-extracted Whole Frog Hearts for Study of Myocardial Fiber Responses.* (21952)

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Since the demonstration by Szent-Györgyi that glycerinated skeletal muscle fibers contract on exposure to adenosine triphosphate, interest has developed in this preparation as a model for the study of muscle contraction (1). Attention has been directed largely to glycerinated psoas muscle fibers because of their length and relative absence of connective tissue (2-4). Glycerol-treated cardiac muscle fibers are more difficult to handle and have not been extensively studied. However, Taeschler and Bing (5) were able to prepare and observe glycerol-extracted trabecular fibers of dog hearts. The purpose of this communication is to describe a glycerinated frog-heart preparation which is suitable for the study of contraction-relaxation cycles.

Method. The frog heart has no coronary circulation, so that tissue nutrition depends upon the diffusion of solutes through the endocardium. The quantity of diffusion is enhanced by numerous fissures in the ventricular walls (6). Therefore, thorough glycerine extraction of the whole heart should be achieved by perfusion and immersion in a glycerol solution. After extraction, ventricu-

lar perfusion with suitable solutes should produce contraction of the muscle fibers. Large bullfrogs (*Rana catesbiana*) are employed. For the preparation of glycerinated hearts, the central nervous system is destroyed and the thorax opened ventrally. The pericardium is excised and the heart is dissected from the mediastinum, care being taken to avoid injury to the ventricle. The fresh heart is placed in Ringer's fluid at room temperature and allowed to beat for several minutes until largely cleared of blood. The heart is then tied to an applicator stick by a ligature tightly drawn around the aortae and upper portion of the atria. A small wire hook is inserted through the ventricular muscle near the apex; the free end of the hook is tied to the stick so as to apply a slight stretch to the ventricle. A needle introduced through the atrium permits the infusion of 50% glycerol into the heart until it is moderately distended. This procedure removes all blood, and the tissue becomes translucent. The mounted specimen is then placed in a capped test tube containing 50% glycerol and is stored at -30 to -10°C for 48 to 72 hours. A thin coat of ice may form at the glycerol-air interface; the tissue should be kept below this level.

Results. After extraction for 2 to 3 days, contraction and relaxation of the glycerinated hearts may be produced by using the perfu-

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[†] National Heart Institute Fellow in Cardiology, 1954-55.

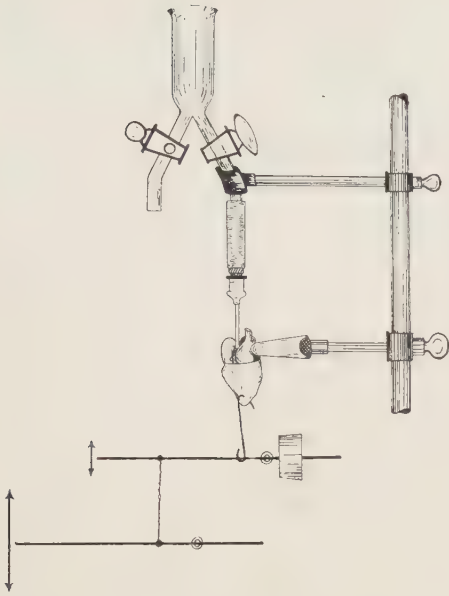


FIG. 1. Diagrammatic sketch of perfusion apparatus with glycerinated frog heart in place. Rate of perfusion of "saline" may be regulated from 30 ml funnel by the stopcock. The counterweighted compound lever system has a net wt of about 800 mg; the system is readily moved by the ventricular muscle fibers.

sion procedure. For this purpose a simple apparatus is employed (Fig. 1). The stick is cut above and below the atrial ligature and the tie holding the hook is severed. The heart is suspended in the apparatus by a pinch clamp applied to the atrium adjacent to the AV groove. The lower end of the hook engages a compound lever system the movements of which are plotted on BMR paper mounted on a kymograph. Writing levers or smoked drums may be used if desired. The heart is perfused by means of a 15-gauge needle inserted through the atrium into the ventricular cavity; the fluid escapes from the ventricle through the small orifice made by the hook. Thorough perfusion of the entire ventricle must be obtained in order to induce maximum contractions. We have found that adequate distention and uniform perfusion of the specimen is assured when the needle is placed through the atrium medial to the bulbus cordis, and a few millimeters into the ventricular chamber. Moderate distention of the tissue probably enhances diffusion of solutes around muscle fibers, and facilitates

contraction by stretch of the fibrils(5). The glycerinated myocardium is friable and caution must be observed to prevent tearing of the tissue.

A solution of 0.15 M KCl and 0.02 M $MgCl_2$ (or other molarities as desired) in glass-distilled water serves as the basic perfusate. This solution is similar to that described by Bozler(4), and is designated as "saline." The suspended heart is irrigated with the "saline" at room temperature for several minutes before the administration of ATP. The di-sodium salt of adenosine triphosphate (Sigma) prepared in "saline" is administered through the perfusion funnel. Concentrations ranging between 0.05% and 4.0% ATP have been used. However, reproducible maximal contractions more often occur with 0.5 to 1.0% ATP solutions. Because of the acidity of ATP, it is our custom to adjust the ATP-saline solution to physiological pH range. The pH adjustment may be carried out with N/10 KOH immediately before use.

More than 130 glycerinated frog heart preparations have been studied using this technic. Marked contraction develops within 1 minute from the beginning of ATP perfusion and peak tension occurs within 3 minutes in most instances. We have repeatedly noted

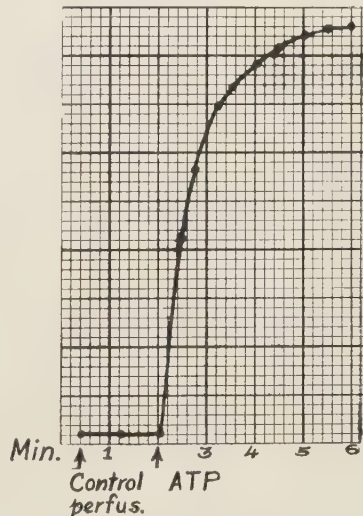


FIG. 2. Example of contraction-curve of a glycerinated whole frog heart. Basic perfusate of KCl .003 M and $MgCl_2$.0004 M. 1% ATP in basic perfusate added at indicated point.

that ATP contractions of the preparations are enhanced by permitting the specimens to come nearly to room temperature before they are detached from the applicator sticks and set up for perfusion.

Comment. This model system may be of value in *qualitative* studies of contraction and relaxation phenomena in glycerinated cardiac muscle. Observations concerning mechanisms of relaxation are in progress. The rapidity of the diffusion of solutes around the muscle cells is confirmed by the rapidity of ATP response. The ease of operation of the model system and the simplicity of preparation commend this technic for experimental observations of myocardial contractile proteins.

Summary. A method is described for the study of contraction-relaxation phenomena in glycerol-extracted cardiac muscle. Excised

hearts of bullfrogs are mounted and extracted in 50% glycerine; subsequently, perfusion with electrolyte solutions and ATP may be carried out, and the contraction recorded by a mechanical system. The ease of preparation and the dependability of the muscle model may prove to be of value in the study of glycerol-extracted heart muscle.

1. Szent-Györgyi, A., *Biol. Bull.*, 1949, v96, 140.
2. Korey, S., *Biochim. and Biophys. Acta*, 1950, v4, 58.
3. Ranney, R. E., *Am. J. Physiol.*, 1954, v178, 99.
4. Bozler, E., *ibid.*, 1951, v167, 276.
5. Taeschler, M., and Bing, R. J., *Circulation Res.*, 1953, v1, 129.
6. Holmes, Samuel J., *Biology of the Frog*, 4th Ed. New York, Macmillan Co., 1927.

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Effect of Lipemia and Heparin on Free Fatty Acid Concentration of Serum in Humans.* (21953)

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The discovery that the *in vitro* clearing of lipemia by plasma obtained after injection of heparin involves lipolysis of triglyceride with release of free fatty acid (1-5) has directed attention to the possibility that free fatty acid might be released *in vivo* by the action of post-heparin clearing factor. Furthermore, the possibility that clearing factor may participate in normal fat metabolism and transport, even when heparin has not been injected (6-8), suggests that free fatty acid may play a physiological role.

It was shown (9) that in rats the free fatty acid concentration of the serum rose when heparin was injected or when alimentary lipemia was present. The present study extends

these observations to human subjects in whom lipemia was induced both by the alimentary route and by intravenous injection of fat emulsion and in whom heparin was injected intravenously both in the fasting state and during alimentary lipemia.

Subjects, materials and methods. Ten healthy young adult males, conscientious observers assigned to the Metabolic Research Division of this laboratory, served as subjects. Heparin (Testagar, 10 mg/ml) was injected intravenously at a dose of 0.5 mg/kg body weight. For the production of alimentary lipemia commercial cottonseed oil (Wesson) was used. The fat emulsion used for intravenous injection (Lipomul, Upjohn) had the following composition: cottonseed oil 15%, dextrose 4.2%, soybean phosphatide 1%, and polyethoxy-propylene oxide (Pluronic F-68, Wyandotte) 0.5%. *Blood lipid analyses.* Venous blood samples were taken from the antecubital vein in chilled syringes and placed immediately in an ice water bath.

* The opinions expressed in this paper are those of the authors and do not necessarily represent those of any governmental agency.

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TABLE I. Blood Lipid Levels of Human Subjects, Fasting, Lipemic, and after Injection of Heparin.

	Fasting	Lipemic	Lipemic, post-heparin	Fasting, post-heparin
Optical density				
Whole serum				
Mean	.072	.211	.158	
Stand. dev.	.018	.086	.091	
Infranantant				
Mean	.044	.055	.046	
Stand. dev.	.006	.009	.009	
Total fatty acid (meq/l)				
Whole serum				
Mean	14.0	17.7	15.8	
Stand. dev.	4.68	6.81	3.35	
Infranantant				
Mean	12.1	13.1	14.4	
Stand. dev.	1.69	2.30	2.73	
Free fatty acid (meq/l)				
Whole serum				
Mean	.51	.75	1.76	1.00
Stand. dev.	.11	.14	.51	.18
Infranantant				
Mean	.51	.75	2.49	
Stand. dev.	.11	.14	.87	

Refrigeration was maintained during centrifugation and separation of the serum up to the time of analysis. Optical density was determined at 700 $m\mu$ in a Coleman Junior spectrophotometer using microcuvettes. Total fatty acid was determined by a modification of the method of Smith and Kik(10), and free fatty acid was determined by a modification(9) of the method of Davis(11). In the study of alimentary lipemia analyses were done on whole serum and on the infranantant obtained by high speed centrifugation. The high speed centrifugation was carried out in a Servall superspeed centrifuge in a room maintained at 5°C. After 30 minutes centrifugation at 14,000 rpm the infranantant was drawn off with a syringe and needle and recentrifuged in a similar fashion.

Results. Alimentary lipemia and heparin injection. After a 12-hour overnight fast the 10 subjects received 150 ml of cottonseed oil by fine polyethylene nasogastric tube. Four hours later heparin was given intravenously in a dose of 0.5 mg per kg of body weight. Blood samples were drawn immediately before giving the oil (fasting sample), 4 hours later, just before giving the heparin (lipemic sample), and 15 minutes after the heparin injection

(lipemic post-heparin sample). The results are summarized in Table I. The lipemic samples showed an elevation of the values for optical density and free and total fatty acid when compared with the fasting samples. The elevation of free fatty acid concentration was statistically highly significant (Table II). Comparing the values for whole serum and infranantant, it is seen that whereas total fatty acid concentration was significantly reduced by removal of visible turbid lipid, free fatty acid concentration remained unchanged (Table II). Injection of heparin resulted in a decrease in optical density and total fatty acid concentration but a marked elevation of free fatty acid concentration (Table I). The values for free fatty acid concentration of the infranantant samples after heparin injection were even higher than those of the corresponding whole serum. This is attributable to the delay in analysis (about 2 hours) that was required to accomplish the centrifugation. Whole serum post-heparin samples held at 5°C but not centrifuged showed a similar rise in this period of time but pre-heparin samples did not. Thus, in contrast to our previous findings on rat serum(9), refrigeration does not prevent completely *in vitro* lipolysis in human post-heparin serum. For this reason the values for free fatty acid in post-heparin serum given in this paper cannot be taken to represent the true *in vivo* situation since they include an increment that has occurred *in vitro*.

Heparin injection in fasting subjects. Blood samples were taken 15 minutes after intravenous injection of 0.5 mg heparin per kg body weight while the 10 subjects were in the fasting state. Free fatty acid concentration (Table I) was substantially elevated (1.00 meq/l) as compared with the pre-injection control (0.51 meq/l). Because of the possibility that *in vitro* lipolysis was occurring in these post-heparin samples, it cannot be determined how closely this reflects the true *in vivo* situation.

Intravenous injection of fat emulsion. Nine of the test subjects each received 3 separate 500 ml infusions of fat emulsion at 3 different rates on consecutive days. The rates were 30, 60 and 120 drops per minute. The assignment of the order of infusion rates to the subjects

TABLE II. Significance of Differences in Table I.

Difference between	Mean difference	Stand. error of mean difference	t	P
Total fatty acid				
Lipemic, whole serum vs. infranatant	4.58	1.52	3.01	<.01
Free fatty acid				
Fasting, whole serum vs. infranatant	.004	.008	.50	>.05
Lipemic, <i>Idem</i>	.008	.007	1.14	>.05
Whole serum, fasting vs. lipemic	.244	.043	5.67	<.01

followed a Latin square design. Venous blood samples were taken at the end of the infusion.

The results are summarized in Fig. 1. Optical density and free and total fatty acid concentrations bore a linear relation to the logarithm of the infusion rate. Analysis of variance showed that the free fatty acid concentrations were significantly different at the various infusion rates and that the regression of free fatty acid concentration on logarithm of infusion rate did not deviate significantly from linearity. In a separate study it was found that injection of the emulsifying agents without fat did not produce any alteration in optical density or free and total fatty acid concentration(18).

Discussion. The results of the present study confirm the findings of the previous study in rats(9) in showing that an increase in free

fatty acid concentration of the serum occurs during lipemia. In addition it was found that the free fatty acid resides in the non-particulate portion of the serum since its concentration was not reduced by removal of the turbid lipid particles by high speed centrifugation. Serum albumin is known to have a high binding capacity for fatty acid(12-16) and it is probable that most of it is so bound in serum. It has been demonstrated in this Laboratory that when C¹⁴-labeled triglyceride is incubated with post-heparin plasma *in vitro* incorporation of activity into the albumin fraction, as detected by filter paper electrophoresis, occurs(17). The highest concentration of free fatty acid encountered in this study (excluding the heparin studies where *in vitro* lipolysis occurred) was 1.46 meq/l in one of the subjects after rapid intravenous injection of fat emulsion. Assuming a molar concentration of albumin in serum of 0.7 mM/l. this would represent a 2 to 1 molar ratio of fatty acid to albumin. *In vitro* studies (12) have shown that the binding capacity of albumin for fatty acid may go as high as 6 to 1. Although the present study was complicated by the occurrence of *in vitro* lipolysis in the post-heparin samples, it is reasonable to assume that the entire elevation was not due to this factor and that heparin injection in human subjects produces an elevation of *in vivo* free fatty acid concentration comparable to that which has previously been demonstrated in rats(9). Much higher concentrations of total fatty acid were achieved in the studies with intravenous infusion of fat emulsion than in those on alimentary lipemia. It was found that the concentration of free fatty acid rose as the concentration of total fatty acid rose. This suggests that the rate of

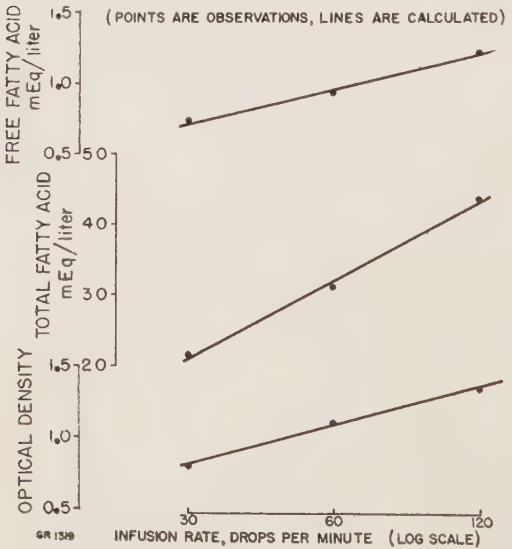


FIG. 1. Relation between rate of infusion of fat emulsion and blood lipid levels at end of infusion in human subjects.

lipolysis is augmented by mass action as the concentration of substrate (triglyceride fat) increases. The findings of this study are consonant with the hypothesis that lipolysis plays a physiological role in removal of turbid fat particles from the blood and that unesterified fatty acid bound to protein may be a mechanism for transporting fat from blood to tissues. Heparin probably accelerates the activity of the lipolytic mechanism(5).

Summary. 1. During alimentary lipemia and lipemia produced by intravenous injection of fat emulsions there was a rise in the concentration of free fatty acid in serum. The free fatty acid was found in the non-turbid portion of serum (infranatant of high speed centrifugation). Injection of heparin produced an elevation of free fatty acid concentration which was greater in lipemic than in fasting subjects. A portion of this elevation after heparin was attributable to *in vitro* lipolysis. 2. It is suggested that lipolysis with free fatty acid formation may play a role in normal fat metabolism and transport.

1. Nichols, A. V., Freeman, N. K., Shore, B., and Rubin, L., *Circulation*, 1952, v6, 457.

2. Shore, B., Nichols, A. V., and Freeman, N. K., *PROC. SOC. EXP. BIOL. AND MED.*, 1953, v83, 216.

3. Nichols, A. V., Rubin, L., and Lindgren, F. T., *ibid.*, 1954, v85, 352.

4. Grossman, M. I., Stadler, J., Cushing, A., and Palm, L., *PROC. SOC. EXP. BIOL. AND MED.*, 1955, v88, 132.

5. Korn, E. D., *Science*, 1954, v120, 399.

6. Bragdon, J. H., and Havel, R. J., *Am. J. Physiol.*, 1954, v177, 128.

7. Levy, S. W., and Swank, R. L., *J. Physiol.*, 1954, v123, 301.

8. Pierce, F. T., *Metabolism*, 1954, v3, 142.

9. Grossman, M. I., Palm, L., Becker, G. H., and Moeller, H. C., *PROC. SOC. EXP. BIOL. AND MED.*, 1954, v87, 312.

10. Smith, M. E., and Kik, M. C., *J. Biol. Chem.*, 1933, v103, 391.

11. Davis, B. D., *Arch. Biochem.*, 1947, v15, 351.

12. Gordon, R. S., Jr., Boyle, E., Brown, R. K., Cherkes, A., and Anfinson, C. B., *PROC. SOC. EXP. BIOL. AND MED.*, 1953, v84, 168.

13. Klotz, I. M., and Ayers, J., *Trans. Faraday Soc.*, 1953, v13, 189.

14. Robinson, D. S., and French, J. E., *Quart. J. Exp. Physiol.*, 1953, v38, 233.

15. Teresi, J. D., and Luck, J. M., *J. Biol. Chem.*, 1952, v194, 823.

16. Davis, B., and Dubos, R., *Arch. Biochem.*, 1946, v11, 201.

17. McDaniel, R. A., and Grossman, M. I., *PROC. SOC. EXP. BIOL. AND MED.*, 1955, v89, 442.

18. Moeller, H. C., Haynes, R. C., Bernstein, L. M., Levy, L. M., and Grossman, M. I., *Med. Nutrition Lab. Rep.* No. 163, 1955.

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Effect of Triiodothyronine on Oxygen Consumption of Tissues Not Responsive to Thyroxine.* (21954)

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The most prominent effect of thyroxine injection in experimental animals is an increase in metabolic rate. Corresponding increases in oxygen consumption have been obtained with such tissues as heart, liver, kidney, diaphragm, skeletal muscle and salivary gland removed from thyroxine-injected animals. In contrast, many other tissues do not participate in the metabolic stimulation, notably brain, spleen, testis, prostate, seminal vesicle, ovary, uterus,

thymus and lymph node(1,2).

Since the discovery of triiodothyronine (TRIT) as a form of thyroid hormone even more potent than thyroxine(3), it has been proposed that the latter may be active only after partial deiodination to triiodothyronine (4). Evidence is accumulating that such a transformation does occur(5,6), although its obligatory character is far from established (7). It seemed possible that the lack of response of the tissues mentioned above, as far as thyroxine was concerned, might be due to

*This work was supported by a grant from the Smith, Kline & French Foundation.

TABLE I. Effect of Injections of L-Thyroxine or of L-Triiodothyronine on Tissue Metabolism of Thyroidectomized Rats.

Tissue	Thyroidect.,* QO ₂	% change from control QO ₂		
		Thyroxine,* 2 mg/kg/d	TRIT,* .5 mg/kg/d	TRIT,† 2 mg/kg/d
Liver	1.02	+ 42.2	+ 44.1	+ 63.8
Diaphragm	.62	+ 43.2	+ 32.3	+ 47.7
Salivary gland	1.98	+ 24.7	+ 37.9	+ 49.1
Heart	.58	+132.4	+135.9	+200.3
Brain	1.34	- .7	- 1.5	+ 2.4
Spleen	1.42	- 4.9	+ 5.6	+ 1.6
Testis	.62	+ 12.9	+ 8.1	- 5.7
Seminal vesicle	.89	+ 1.1	- 4.5	- 3.8
Prostate	1.45	+ 6.2	- 6.2	+ 4.0
Ovary	1.49	+ 4.7	+ 3.4	+ 1.7
Uterus	1.12	- 11.6	- 14.3	+ 7.7
Thymus	1.14	+ 5.3	+ 3.5	+ 4.8
Lymph node	1.17	- .9	+ 11.1	+ 2.5
Gastric Sm. Mus.	.73	+ 2.7	.0	- 3.9

* Five ♂ and 5 ♀ in each of these groups.

† One ♂ and one ♀ in this group.

inability to carry out this transformation. The results of the present work show that, although TRIT is some 4 times as effective as thyroxine in increasing metabolism of several tissues, its results are not qualitatively different from those of thyroxine.

Methods. Both male and female albino rats of the Sprague-Dawley strain were used. All were thyroidectomized at least 4 weeks prior to being injected. As a reference material, L-thyroxine† was injected in a dose of 2 mg/kg/day for 4 days and the animals sacrificed on the fifth day. Preliminary studies indicated that an equimolar dose of L-triiodothyronine‡ produced a much greater elevation in metabolism of heart, liver, diaphragm and salivary gland than resulted from thyroxine. Since one-quarter of the equivalent dose gave approximately the same effect as thyroxine, this amount was used. The compounds to be injected were dissolved in a small amount of 0.1 N NaOH, which was diluted to a final concentration of 0.02 N and was made isotonic with NaCl. Some of the control animals were injected with an equivalent amount of the alkaline saline; since no differences were observed from the uninjected, all control animals

were lumped together. Preparation of each of the various tissues was carried out as discussed previously(1,2). Oxygen consumption of each tissue was measured in a Krebs' Ringer phosphate medium with glucose (100 mg%) as substrate.

Results. Table I shows the average oxygen consumptions for the array of tissues studied from the control thyroidectomized animals and from those injected with thyroxine and triiodothyronine. Five males and 5 females were used in each category of control and thyroxine-injected or TRIT-injected. The averages of all tissues not sex-specific are therefore from 10 animals. It can be seen that, amongst the tissues used as references, liver and heart were increased about the same amount by both substances at the dose levels employed. Triiodothyronine accelerated salivary gland metabolism somewhat more, and diaphragm less than did thyroxine. Two additional rats are included which received 4 times as much triiodothyronine as the others, or a dose equimolar with the thyroxine. The oxygen consumption increases were greater in these 2, but not proportionally.

The remaining data in the Table deal with tissues previously found unresponsive to thyroxine, with one exception. These were again unaltered by thyroxine and in addition were not changed by either the 0.5 or the 2.0 mg dose of triiodothyronine. The one exception

† L-Thyroxine was obtained from the Travenol Laboratories, through the kindness of Dr. H. A. Evold.

‡ L-Triiodothyronine was furnished by Dr. A. E. Heming of Smith, Kline & French Laboratories.

was gastric smooth muscle which had previously been reported as stimulated by thyroxine injection(1). When neither TRIT nor thyroxine influenced its metabolism in the present series, the original data of Barker and Klitgaard were carefully re-examined, but no explanation could be discovered for the discrepancy. Preliminary experiments with several other thyroxine analogs have yielded results similar to those shown here. This tissue now becomes consistent with other unresponsive structures also made up primarily of smooth muscle, such as uterus and seminal vesicle.

One may conclude from these data that, in the thyroidectomized rat, triiodothyronine produces qualitatively the same effects on tissue metabolism as thyroxine. As already mentioned, there is no clear evidence that formation of the triiodinated form from the tetraiodinated is an obligatory step in the tissue response to the latter. The present study discloses that, even if triiodothyronine is made available, some tissues do not respond. An interesting point to pursue would be to determine whether any of these resistant tissues is actually capable of deiodinating thyroxine.

Discussion. Unpublished observations of Klitgaard and Barker indicate that after injection of these amounts of thyroxine, the hormone is present in all tissues to about the same concentration, except for liver and kidney, where levels are much higher. Comparable data are not available for triiodothyronine. At much lower dose levels, both forms of the hormone have been shown to be present in many tissues of the animal body(8). The question of penetration of cells by thyroxine or TRIT has not been answered clearly although Gross *et al.*(8,9). have demonstrated

considerable concentration of TRIT by parts of the brain, especially posterior pituitary.

Summary. 1. L-Triiodothyronine (TRIT) injected into thyroidectomized rats at a dose level equivalent to 0.5 mg L-thyroxine/kg body weight/day is capable of stimulating the oxygen consumption of liver, diaphragm, heart and salivary gland preparations to the same extent as 2 mg of L-thyroxine. 2. Neither 0.5 nor 2 mg TRIT injected/kg/day altered the metabolic rates of tissues found unaffected by 2 mg of L-thyroxine, including brain, spleen, testis, seminal vesicle, prostate, ovary, uterus, thymus, lymph node and gastric smooth muscle. 3. Even if thyroxine is converted to TRIT before exerting its effect on the metabolic rate the failure of these tissues to respond to thyroxine does not seem referable to any possible inability to carry out this deiodination.

1. Barker, S. B., and Klitgaard, H. M., *Am. J. Physiol.*, 1952, v170, 81.
2. Barker, S. B., and Schwartz, H. S., *Proc. Soc. Exp. Biol. and Med.*, 1953, v83, 500.
3. Gross, J., and Pitt-Rivers, R., *Lancet*, 1952, vI, 439, 593; Roche, J., Lissitzky, S., and Michel, R., *Compt. rend. Acad. Sci.*, 1952, v234, 997.
4. Gross, J., and Pitt-Rivers, R., *Biochem. J.*, 1953, v53, 652.
5. Flock, E. V., and Bollman, J. L., *Fed. Proc.*, 1954, v13, 209.
6. Albright, E. C., Larson, F. C., and Tust, R. H., *Proc. Soc. Exp. Biol. and Med.*, 1954, v86, 137.
7. Hogness, J. R., Van Arsdell, P., Jr., and Williams, R. H., *J. Clin. Endocrinol. and Metab.*, 1954, v14, 772.
8. Gross, J., in *The Thyroid, Brookhaven Symposia in Biology*, 1955, No. 7, 102.
9. Gross, J., Ford, D. H., and Posner, M., *J. Clin. Endocrinol. and Metab.*, 1955, v15, 836.

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Comparison Between Changes in Serum Inorganic Phosphorus Induced by Glucose and Glucagon in Diabetics. (21955)

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Kirtley, Waife and Peck(1) first reported the effect of glucagon upon blood sugar and serum inorganic phosphate in diabetics. In 4 "stable" diabetics, blood sugar rose to approximately the same level as in normal subjects, remained elevated longer and the serum inorganic phosphorus fall was impaired. In 4 "unstable" diabetics, there was a lesser blood sugar increase and the return to basal levels was about the same as in normals; phosphorus fall was the same as in normals in this group. Kirtley *et al.*(2) extended these observations and showed with epinephrine results similar to those obtained with glucagon.

In the following investigation, we wished to inquire whether diabetics, classified according to the Delta G/Delta P test (based on magnitude of serum inorganic phosphorus fall after intravenous injection of glucose) presented differences in regard to response to glucagon.

Material and methods. The tests were carried out in 35 diabetic patients chosen at random, most of them without previous insulin treatment. A few of the subjects, who were being treated with long acting insulin prepara-

tions, were given regular insulin instead for the 2 days preceding the tests. The patients were kept fasting from the night preceding the test and remained at rest for a half hour before and during the test. The Delta G/Delta P test was performed according to a previously described technic, consisting essentially in the intravenous injection of 1 ml/kg body weight of a 50% glucose solution at the rate of approximately 10 ml/m; samples of blood are taken at 0, 30, 45 and 60 minutes. Blood sugar was determined by the Nelson-Somogyi method(3) and serum inorganic phosphorus by the Fiske-Subbarow method(4), modified for the spectrophotometer. According to previous investigations(5,6), an increase in blood sugar at 45 minutes over the initial level (Delta G) of more than 50 mg/100 ml is evidence of a decrease in the subject's capacity to remove glucose from the blood stream, and a decrease in serum inorganic phosphorus below the initial level at 45 minutes (Delta P) of less than 0.30 mg/100 ml is considered defective. A normal Delta P is presumptive evidence of insulin secretion.

TABLE I. Changes in Serum Inorganic Phosphorus (mg/100 ml) after Administration of Glucose in Diabetics. Limiting value between the two groups has been taken as -0.30 mg/100 ml decrease from the initial value at 45 min.

Averages and standard errors.

Group	No. of cases	Initial values	30'	45'	60'
Defective Δ P	19	3.14 \pm .13	-.07 \pm .04	-.09 \pm .01	-.06 \pm .02
Normal Δ P	16	3.42 \pm .22	-.31 \pm .01	-.50 \pm .04	-.45 \pm .06

TABLE II. Changes in Blood Sugar after Administration of Glucose (mg/100 ml) in Diabetics Classified According to Serum Inorganic Phosphorus Fall in the Same Test.

Averages and standard errors.

Group	No. of cases	Initial values	30'	45'	60'
Defective Δ P	19	236 \pm 18	+143 \pm 7	+112 \pm 8	+87 \pm 7
Normal Δ P	16	133 \pm 12	+131 \pm 9	+ 96 \pm 9	+75 \pm 9
"t" values		4.8*	1.1	1.3	1.1

* Statistically significant difference.

*The author is grateful for the technical assistance

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TABLE III. Changes in Serum Inorganic Phosphorus (mg/100 ml) after Glucagon in Diabetics Classified According to Phosphorus Fall after Glucose.
Averages and standard errors.

Group	No. of cases	Initial values	15'	30'	45'	60'
Defective ΔP	19	$3.08 \pm .14$	$.47 \pm .07$	$.53 \pm .07$	$-.40 \pm .05$	$.30 \pm .04$
Normal ΔP	16	$3.37 \pm .13$	$-.57 \pm .02$	$.72 \pm .02$	$-.54 \pm .09$	$-.39 \pm .14$
"t" values		1.5	1.3	2.8*	1.4	.7

* Statistically significant difference.

TABLE IV. Changes in Blood Sugar (mg/100 ml) after Glucagon in Diabetics Classified According to Their Phosphorus Fall after Glucose.
Averages and standard errors.

Group	No. of cases	Initial values	15'	30'	45'	60'
Defective ΔP	19	232 ± 18	$+32 \pm 5$	$+46 \pm 3$	$+48 \pm 5$	$+42 \pm 6$
Normal ΔP	16	137 ± 14	$+35 \pm 2$	$+54 \pm 3$	$+53 \pm 4$	$+42 \pm 5$
"t" values		4.1*	.5	1.8	.7	0

* Statistically significant difference.

The patients were classified according to their Delta P and were then administered glucagon solution in a fashion similar to the administration of glucose; 0.01 ml of glucagon solution per kg body weight was given intravenously, without dilution, and an additional 15 minute sample of blood was obtained. According to specifications given by the manufacturer, the glucagon solution which was used (lot 208-158B-214) contained 0.95 mg of protein/ml, with 0.25% phenol, pH 3. Purity, as compared to crystalline glucagon was 50%; 0.2 μ g per kg injected intravenously to an anesthetised cat caused a maximum increase in blood sugar of 30-40 mg per 100 ml 10 to 15 minutes after the injection. The content of insulin was between 0.005 and 0.05 U/ml of solution. The solution was generously furnished by the Eli Lilly Co.

Results. In Table I are shown the changes in serum inorganic phosphorus in the group of diabetics studied, divided according to the fall following glucose.

In Table II may be seen the changes in blood sugar after glucose in both groups.

There is no statistically significant difference with respect to blood sugar rise following glucose between the two groups. The initial level was higher in the group with defective Delta P.

Table III demonstrates serum inorganic phosphorus response to glucagon in the groups

classified according to their Delta P after glucose. At 30 minutes, there is a statistically significant difference between the 2 groups,

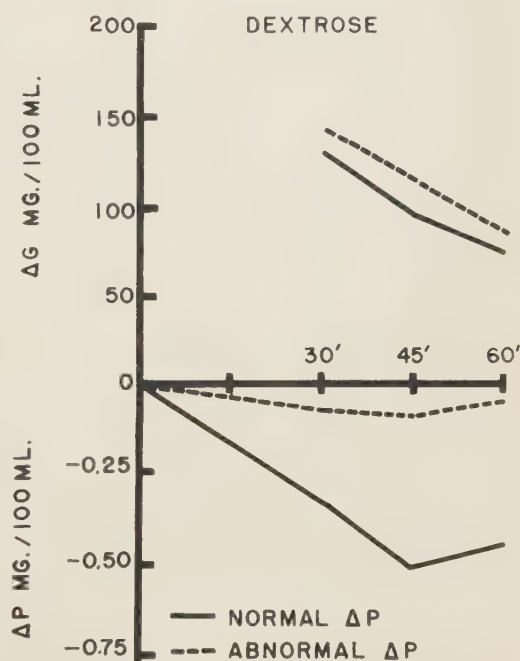


FIG. 1. Changes in blood sugar (ΔG) and in serum inorganic phosphorus (ΔP) after intravenous administration of dextrose in diabetics classified according to their phosphorus fall in the same test (limiting value between the 2 groups = -0.30 mg/100 ml).

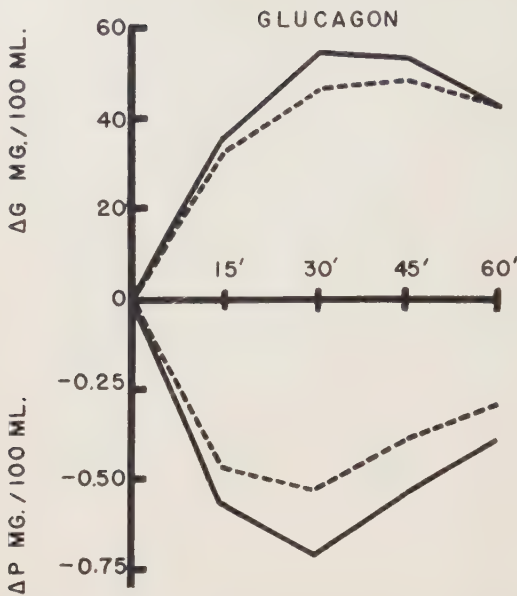


FIG. 2. Changes in blood sugar and serum inorganic phosphorus after intravenous inj. of a glucagon solution in diabetics classified according to their serum inorganic phosphorus response to dextrose. The groups are the same as those indicated in Fig. 1.

the group with "normal" phosphate fall after glucose showing a greater phosphate fall after

glucagon. In Table IV the changes of blood sugar after glucagon in both groups are shown. The results are summarized in Fig. 1 and 2.

In Fig. 3 is shown the correlation between maximal Delta P produced by glucagon and maximal Delta P determined by glucose. No definite correlation between the two sets of values appears to exist.

Discussion. The serum inorganic phosphorus fall after glucagon is in general agreement with that following glucose; the fall after glucagon is lesser in the abnormal Delta P group, particularly in the 30-minute sample, where the difference is statistically significant.

The fact that glucagon is able to produce a serum inorganic phosphorus fall in the group where such a fall does not occur after glucose is interesting. It is not likely that this effect is due to insulin contamination, since each cc of the solution contains only between 0.05 and 0.005 U/ml and the amount injected is 0.01 ml per kg. The phosphorus falls found with glucagon are comparable or greater than those found in diabetics after 0.1 U of insulin per kg(7). It would then seem that glucagon has an action of its own upon

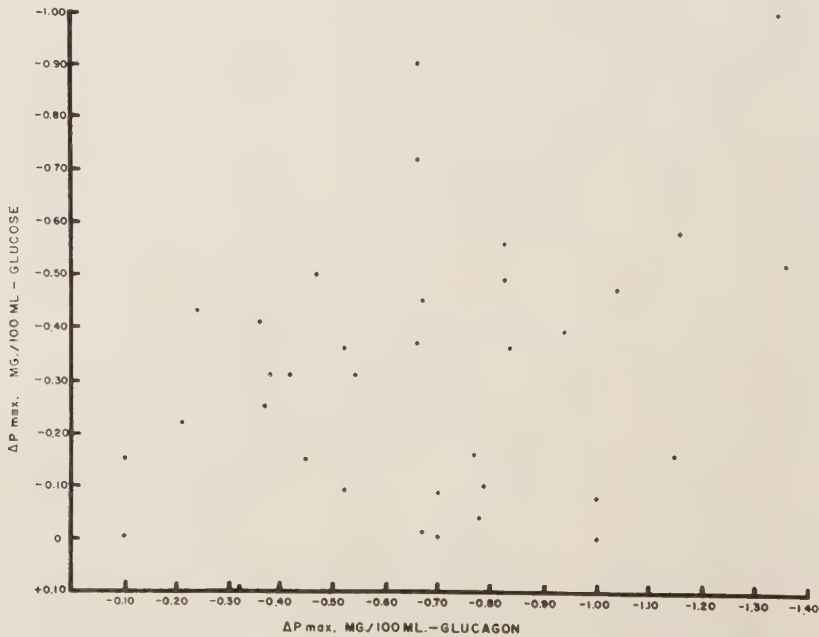


FIG. 3. Correlation graph between maximal serum inorganic phosphorus fall induced by dextrose and by glucagon.

serum inorganic phosphorus, independent from that determined by secondary insulin liberation. Speculatively, it may be thought that this effect depends upon the utilization of phosphate in the disintegration of glycogen by phosphorylase.

Summary. The effect of intravenous glucose upon serum inorganic phosphorus was compared to that of glucagon in 35 diabetic patients taken at random.

The subjects were classified in two groups according to their serum phosphate fall following glucose. In a group of 19 patients, the phosphate fall after glucose was less than 0.30 mg/100 ml, and in another group of 16 patients, more than 0.30 mg/100 ml similar to that found in non-diabetic individuals. As a working hypothesis, it has been assumed that diabetics with a normal phosphate fall are producing insulin. It was found that the group with a normal phosphate fall after glucose showed a greater fall after glucagon than the group with defective phosphate fall. On the other hand, the group with defective or

absent phosphate fall after glucose also showed a very definite fall after glucagon. It would seem then that the latter substance exerts a direct effect upon serum inorganic phosphate, independent of the reactive insulin secretion produced by the glucose load. This effect is possibly related to the activation of phosphorylase which acts in the process of disintegration of glycogen through the introduction of phosphate in the bonds existing between the hexose molecules.

1. Kirtley, W. R., Waife, S. O., and Peck, F. B., *PROC. SOC. EXP. BIOL. AND MED.*, 1953, v83, 387.
2. Kirtley, W. R., Waife, S. O., Helmer, O. M., and Peck, F. B., *Diabetes*, 1953, v2, 345.
3. Nelson, N., *J. Biol. Chem.*, 1944, v153, 375.
4. Fiske, C. H., and Subbarow, Y., *ibid.*, 1925, v66, 375.
5. De Venanzi, F., and Masin, F., *Acta Cientif. Venezolana*, 1950, v2, 214.
6. De Venanzi, F., *ibid.*, 1952, v3, 214.
7. De Venanzi, F., Roche, M., and Vera, J., *PROC. SOC. EXP. BIOL. AND MED.*, 1954, v87, 16.

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Anti-Inflammatory Activity of Δ^1 -9 α -Fluorohydrocortisone Acetate. (21956)

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There have been many important advances in the use of steroids as a method of treatment of the inflammatory diseases during the past few years. Since the introduction of hydrocortisone as one of the most efficacious treatments, it has been continually modified with the hopes of increasing its antiphlogistic activity while diminishing its undesirable side effects. It has been found that halogenation at the C-9 position greatly increases the glycogen deposition potency(1,2) and the anti-inflammatory effects(3) but causes a relatively greater increase in mineralocorticoid activity (4,5,6). A second analogue of hydrocortisone, 1-dehydrohydrocortisone, has been reported to possess greater antiarthritic properties in man (7) than the parent compound. It has also been shown that this compound is a more po-

tent glucocorticoid in animals(8,9) and that this increase is not associated with an increase in mineralocorticoid potency(8).

It was, therefore, of interest to study the effects of both of these modifications when introduced into the same molecule. Accordingly, the anti-inflammatory activity of Δ^1 -9 α -fluorohydrocortisone acetate was compared with that of hydrocortisone. Data are also included showing the relative potencies of Δ^1 -hydrocortisone acetate, 9 α -fluorohydrocortisone acetate, and hydrocortisone for comparative purposes.

Materials and methods. Animals. Male rats obtained from the Upjohn colony (Sprague-Dawley ancestry) were used throughout these experiments. The body weights ranged from 150-170 g. All animals

were adrenalectomized and allowed free access to Archer dog pellets and 1% sodium chloride drink. They were housed in small wire cages with 4 animals per cage. *Steroids.* The steroids were injected subcutaneously as a suspension in a vehicle containing 0.5% carboxymethyl cellulose, 0.4% Tween 80, 1.5% benzyl alcohol, and 0.9% sodium chloride. The suspensions were made by grinding the crystalline steroids plus vehicle in a ground glass tissue homogenizer. The compounds* used were: (1) 11 β , 17 α , 21-trihydroxy-4-pregnene-3,20-dione (hydrocortisone), (2) 9 α -fluoro-11 β , 17 α , 21-trihydroxy-4-pregnene-3,20-dione, 21-acetate (9 α -fluorohydrocortisone acetate), (3) 11 β , 17 α , 21-trihydroxy-1,4-pregnadiene-3,20-dione, 21-acetate (Δ^1 -hydrocortisone acetate), and (4) 9 α -fluoro-11 β , 17 α , 21-trihydroxy-1,4-pregnadiene-3,20-dione, 21-acetate (Δ^1 -9 α -fluorohydrocortisone acetate). *Anti-Inflammatory Test.* The test used was a modification of the cotton pellet test as described by Meyer, *et al.* (11). It consisted essentially of implanting 2 nonsterile cotton dental pellets, weighing 6-8 mg, in the mid ventro-lateral subcutaneous connective tissue through a small incision in the skin which was closed by a metal wound clip. The implanting was done at the time of adrenalectomy while the animals were under anesthesia. Anesthesia was accomplished by intraperitoneal injection of sodium pentobarbital at a dosage of 40 mg per kg. Seven days after implantation, the pellets, with their accumulated granulation tissue, were dissected out, dried at 60°C for 24 hours, and weighed to the nearest 0.1 mg on a Roller-Smith Torsion balance. The original cotton weight was subtracted from the final value in order to get the net amount of granulation tissue. The injection of the compounds was begun at the time of implantation and continued through seven daily injections. The volume of each injection was 0.2 cc. The animals were sacrificed approximately 24 hours following the last injection. The experiments were designed so that each steroid was tested

at two dosage levels in parallel with hydrocortisone which was used as the standard. The potency ratios were calculated by the method of Irwin (10) and are expressed in terms of activity of hydrocortisone, which is assigned a value of 1. The data on Δ^1 -9 α -fluorohydrocortisone acetate were pooled from 3 separate experiments and the data on Δ^1 -hydrocortisone acetate were pooled from 2 experiments.

Results. The comparison of the antiphlogistic potency of Δ^1 -hydrocortisone acetate, 9 α -fluorohydrocortisone acetate, and Δ^1 -9 α -fluorohydrocortisone acetate with hydrocortisone is summarized in Table I. These data show that the potency ratio for Δ^1 -hydrocortisone acetate is 3.1, for 9 α -fluorohydrocortisone acetate 7.3, and for Δ^1 -9 α -fluorohydrocortisone 14, whereas hydrocortisone is assigned a value of 1.

Discussion. It has been reported (7) that Δ^1 -hydrocortisone is 2 to 3 times as effective as hydrocortisone in the treatment of arthritis. Others (8,12) have found that it is 3.0 to 3.9 times as active as hydrocortisone by the liver glycogen deposition test. The anti-inflammatory test, as described here, shows that Δ^1 -hydrocortisone acetate is 3.1 times as active as the parent compound. These results, therefore, are in good agreement with the clinical data and results on glycogen deposition.

The activity of 9 α -fluorohydrocortisone acetate has been found to be 12 times hydrocortisone by the glycogen deposition test (12) and 13 times hydrocortisone acetate by an anti-inflammatory test similar to the one described in this paper (3). It is interesting to note that the data reported here show that this halogenated compound is 7.3 times as active as hydrocortisone.

It is seen by examination of the data in Table I that Δ^1 -9 α -fluorohydrocortisone acetate is 14 times as effective as hydrocortisone. It was found by Stafford, *et al.* (12) that it was approximately 50 times as active as hydrocortisone on glycogen deposition. Therefore, an apparent quantitative discrepancy exists between the classical glucocorticoid activity and antiphlogistic effect. This could be due to at least two possible reasons: (1) glycogen deposition and anti-inflammatory ef-

* The compounds used in this study were kindly supplied by the Departments of Biochemistry and Chemistry, Research Division, The Upjohn Co.

TABLE I. Anti-Inflammatory Assays of Hydrocortisone, 9 α -Fluorohydrocortisone Acetate, Δ^1 -Hydrocortisone Acetate and Δ^1 -9 α -Fluorohydrocortisone Acetate.

Treatment	Dose	No. rats	Mean dry granuloma wt (mg) \pm S.E.	Potency (\times hydrocortisone)
CMC	0.2 cc	20	14.41 \pm .77	
Hydrocortisone	200 μ g	17	9.97 \pm .46	
	1000 "	16	6.57 \pm .34	1
Δ^1 -9 α -Fluorohydrocortisone acetate	10 "	20	9.91 \pm .46	
	50 "	19	7.90 \pm .47	14
CMC	0.2 cc	7	13.40 \pm .47	
Hydrocortisone	200 μ g	7	9.80 \pm 1.25	
	1000 "	6	6.32 \pm .29	1
9 α -Fluorohydrocortisone acetate	25 "	7	10.74 \pm .34	
	125 "	8	6.05 \pm .44	7.3
CMC	0.2 cc	10	12.20 \pm .49	
Hydrocortisone	200 μ g	8	10.10 \pm .65	
	1000 "	9	5.70 \pm .42	1
Δ^1 -Hydrocortisone acetate	60 "	10	10.00 \pm .77	
	300 "	10	6.20 \pm .50	3.1

fects are mediated through different mechanisms, or (2) the nature of the tests are such that differences in physical properties of the compounds, such as solubilities, could affect the results of the two tests in a quantitatively different manner.

The fact that Δ^1 -9 α -fluorohydrocortisone acetate has an anti-inflammatory activity greater than the additive effects of Δ^1 -hydrocortisone acetate and 9 α -fluorohydrocortisone acetate is of significance. The mechanism responsible for the increase in activity produced by introducing two important chemical changes into the same molecule is not clear at present but is worthy of further investigation.

Summary. A comparison of anti-inflammatory effects of hydrocortisone with several of its analogues has been made. It was found that Δ^1 -hydrocortisone acetate was 3.1 times as active as hydrocortisone; 9 α -fluorohydrocortisone acetate was 7.3 times as active, while Δ^1 -9 α -fluorohydrocortisone acetate was 14 times as effective as hydrocortisone, as determined by the cotton pellet implantation method.

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1. Fried, J., and Sabo, E., *J. Am. Chem. Soc.*, 1953, v75, 2273.
2. ———, *ibid.*, 1954, v76, 1455.
3. Singer, F. M., and Borman, A., *Fed. Proc.*, 1955, v14, 281.
4. Borman, A., Singer, F. M., and Numerof, P., *Proc. Soc. Exp. Biol. and Med.*, 1954, v86, 570.
5. Swingle, W. W., Baker, C., Eisler, M., LeBrie, S. J., and Brannick, L. F., *ibid.*, 1955, v88, 193.
6. Liddle, G. W., Pechet, M. M., and Bartter, F. C., *Science*, 1954, v128, 496.
7. Bunim, J. J., and Pechet, M. M., *J.A.M.A.*, 1955, v157, 311.
8. Perlman, P. L., and Tolksdorf, S., *Fed. Proc.*, 1955, v14, 377.
9. Herzog, H. L., Nobile, A., Tolksdorf, S., Charney, W., Hershberg, E. B., and Perlman, P. L., *Science*, 1955, v121, 176.
10. Irwin, J. O., *Suppl. J. Royal Stat. Soc.*, 1937, vIV, 1.
11. Meyer, R. K., Stucki, J. C., and Aulsebrook, K. A., *Proc. Soc. Exp. Biol. and Med.*, 1953, v84, 624.
12. Stafford, R. O., Barnes, L. E., Bowman, B. J., and Meinzinger, M. M., *ibid.*, 1955, v89, 371.

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Fluorescent Antibody and Complement-Fixation Tests of Agents Isolated In Tissue Culture from Measles Patients.* (21957)

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Enders and Peebles(1) observed that human or monkey kidney tissue cultures inoculated with specimens from measles patients undergo, after one or more passages, characteristic nuclear and other cytologic changes. Both infectious virus and specific complement-fixation antigen appeared concurrently in the cultures and the cytopathogenic effect was neutralized by convalescent-phase measles serum. We have applied the fluorescent antibody technic of Coons and his colleagues(2,3) to similar cultures and find that the results parallel those of complement-fixation tests and thus provide immunochemical support for the data of Enders and Peebles and in addition evidence that measles antigen(s) are present in the nuclei as well as in the cytoplasm of infected cells.

Methods. Patients were selected for study during a mild outbreak of typical rubeola. Clinical diagnosis was aided in certain cases by recognition of giant cells of the Warthin-Finkeldey type in stained films of nasal discharge obtained early in illness, as described by Tompkins and Macaulay(4). *Collection of specimens and preparation of tissue cultures.* The procedures outlined by Enders and Peebles(1) were followed. Blood, throat swabs or washings, or nasal discharge were obtained before or within a day after the appearance of the rash. When possible, specimens were inoculated into tissue cultures

within a few hours after their collection. Tissue cultures consisted of trypsinized monkey kidney cells grown in sheets(5). Tubes were inoculated with 0.2 or 0.3 ml of the prepared specimens, fed with nutrient fluid to bring the volume to 1.0 ml, and incubated as stationary slants at 36.5°C. Nutrient fluid consisted of beef amniotic fluid containing 5% inactivated horse serum and 5% beef embryo extract. The first passage contained 100 units of penicillin, 100 µg of streptomycin, and 50 µg of nystatin (fungicidin) (6); others, 50 units and 50 and 10 µg, respectively. Transfers to fresh tubes were carried out at 14-day intervals with 0.2-ml amounts of pooled fluids taken 5, 9, and 14 days after inoculation. Controls consisted of uninoculated cultures and of cultures passaged serially with fluids from uninoculated tubes. *Staining with fluorescein-labeled antibody.* The monkey kidney cells were gently scraped from the tube walls, or sedimented by centrifugation of the tissue culture fluid, washed with NaCl buffered at pH 7.6(7), sedimented, and spread on slides. The films were dried in air and fixed with acetone. They were often stored at 4°-6°C for from 1 to 3 days before staining by the indirect method of Weller and Coons(3). The test serum diluted 1:20 was acute- or convalescent-phase measles or heterologous human serum. Fluorescent antibody was prepared from antihuman gamma globulin horse serum generously supplied by Dr. David Gitlin, Children's Medical Center, Boston. The pseudoglobulin fraction was conjugated with fluorescein isocyanate, dialyzed, and stored at -20°C. Before use it was absorbed with mouse liver powder to remove nonspecific staining(2), then diluted 1:10. Prior to staining with the fluorescent antibody solution, films of uninoculated and inoculated preparations were treated, one of each with acute-, the other with convalescent-phase measles serum, to control the staining

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technics. The equipment used for fluorescence microscopy has been described by Buckley, Whitney, and Rapp(8). *Complement-fixation antigens* consisted of the fluid phase from tissue cultures in tubes or in 200-ml square bottles. For the latter, 1-ml amounts of inoculum and 7 ml of nutrient fluid were used. Medium 703(9) with or without 5% inactivated horse serum was substituted for Enders' medium(1) in some lots with satisfactory results. The fluids were centrifuged at about 2500 r.p.m. for 15 minutes and inactivated at 56°C for 30 minutes. *Complement-fixation tests* were made with three 50% units of complement. Fixation was at 4°C for 24 hours. For serum titrations, 2-fold dilutions were tested against a previously determined optimum dilution of antigen. Titers were the calculated number of 50% units of complement fixed by undiluted serum.

Results. Transmissible agents, presumably viruses, were isolated from both blood and a throat swab of one patient, and from nasal discharge and a throat swab, respectively, of 2 others whose blood was not obtained. The throat swab specimens had been stored in a dry ice chest for 7 days. No agents were detected in specimens from 5 additional cases. Inocula from 3 of the latter had been stored at 4°-6°C for 2 days before culture; tests of inocula from the other 2 were discontinued after 3 apparently negative serial passages. These circumstances may have contributed to our failure to isolate agents.

Enders and Peebles(1) and Rustigian *et al.* (10) encountered latent virus-like agents that induce marked vacuolization and syncytial masses in monkey kidney tissue cultures. The cellular degeneration characteristic of these "monkey-kidney agents" frequently appeared in our cultures, both in those inoculated with specimens from measles patients and in controls; hence cytologic criteria for recognition of measles agents were difficult to apply. In some tissue culture series the "monkey-kidney agents" destroyed the cell sheets in 10 to 14 days. We therefore relied on tests for the presence of measles antigen to identify the 3 agents cultivated from measles inocula.

The reactivity of the measles agents in both fluorescent antibody and complement-

fixation tests seemed to be specific. Cells from control monkey kidney cultures, some with extensive "foamy" areas and multinucleated cells, did not exhibit fluorescence nor did control tissue culture fluids fix complement with either acute- or convalescent-phase sera from measles cases. Cells infected with measles agents, however, were brightly fluorescent with the 3 measles convalescent-phase sera tested but not with 3 acute-phase sera or with human antisera to herpes simplex, influenza Types A and B, or mumps viruses. The proportion of brightly and specifically fluorescent cells increased as serial passages continued. After 4 passages, many of the cells apparently contained measles antigen. One of the strains has been carried through 7 passages.

In tests of infected cells, fluorescence was observed in some cultures as early as the 5th day of incubation (Table I). Earlier tests were not made. Between the 5th and 10th day, fluorescence, when present, was usually confined to the cytoplasm. After about 2 weeks, however, bright yellow-green fluorescent intranuclear masses were frequently seen in fluorescent cells, very rarely in non-fluorescent cells (Fig. 1-3). The masses were surrounded by an unstained dark nuclear zone. Attempts to correlate the appearance of specific fluorescent masses in the nuclei with the

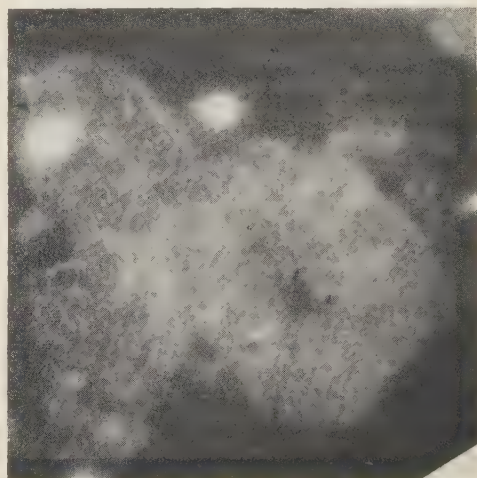


FIG. 1. Monkey kidney cells 14 days after infection with measles agent No. 5584. Indirect fluorescent antibody stain with convalescent-phase measles serum. The lighter areas are those showing specific yellow-green fluorescence. 400X.

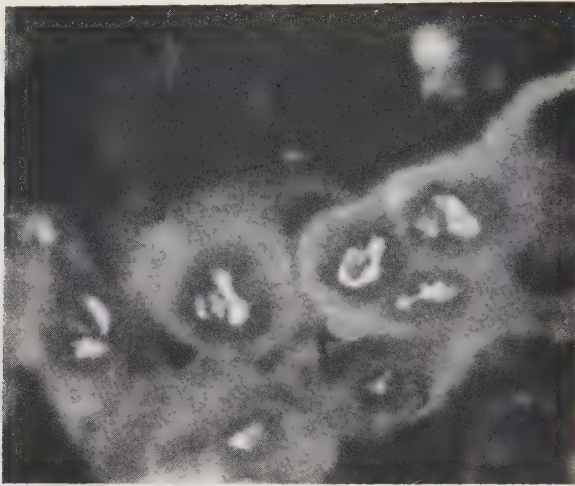


FIG. 2. Monkey kidney cells 13 days after infection with measles agent No. 5585. Stain same as Fig. 1. 700 \times .

intranuclear inclusions described by Enders and Peebles are in progress.

In direct comparisons between development of specific fluorescence and of complement-fixation antigen, the fluorescent antibody procedure seemed the more sensitive of the two methods, since it detected antigen earlier in the course of incubation than did the complement-fixation test (Table I). This difference may have been due to the fact that fluorescent

antibody tests were done on cell spreads, whereas complement-fixation antigens were made from centrifuged fluids. The fluid antigens were relatively weak; they rarely were active when diluted more than twofold. The antigen titer of one fluid taken 25 days after inoculation was considerably increased by addition of an extract of cells from the same cultures. The cells were frozen and thawed for 6 cycles in NaCl solution buffered at pH 7.6; the supernate was the extract.

Only 3 pairs of acute- and convalescent-phase sera could be obtained from measles patients. The complement-fixation titer of each acute-phase serum was <4 ; those of the convalescent-phase specimens were 40, 64, and 200. In tests with 21 sera from 13 cases of measles the distribution of titers agrees with that found by Enders(11) (Table II). Among the 26 heterologous sera tested were convalescent-phase sera from cases of virus infection such as herpes simplex, influenza, and mumps, and unclassified sera received for routine tests for evidence of virus or rickettsial infections. Twenty-three gave relatively low complement-fixation titers.

Discussion. Demonstration that tissue cultures inoculated with specimens from measles patients produce antigens that react specifically with convalescent-phase measles sera substantiates the findings of Enders and



FIG. 3. Control for Fig. 2; same cells stained with acute-phase measles serum. 700 \times .

TABLE I. Correlation of Results of Fluorescent Antibody Tests with Appearance of Complement-Fixation Antigen in Monkey Kidney Tissue Cultures Infected with Measles Agents.

Strain	Tissue culture		Fluorescent antibody results with		CF titer*
	Passage level	Day post-inoculation	Acute-phase serum	Convalescent-phase serum*	
5582, 5583† (blood, throat swab)	3	14	0	+	300
	4	9	0	+	NT
		14	0	+	211
	5	9	0	±	NT
		14	0	+	179
5584 (throat swab)	3	4	NT	NT	<4
		9	NT	NT	>64
		14	0	+	303
	4	10	NT	NT	<8
		14	0	+	<8
		18	NT	NT	<8
		21	0	+	112
	5	14	0	+	<4
	6	5	0	+	<4
		10	0	0	97
		14	0	+	55
		25	0	+	221
5585 (nasal discharge)	1	14	0	±‡	NT
	2	14	0	+	NT
	3	14	0, ±	+	NT
	4	13	±	+	NT
	5	5	0	+	<4
		10	0	0	<4
		14	0	+	<4
		25	0	+	195

CF = complement-fixation. NT = not tested.

Green-yellow fluorescence due to labeled antibody was graded as follows: 0 = none, ± = faint or rare cell bright, + = bright.

* Convalescent-phase serum 55291 used for both procedures.

† Combined blood (5582) and throat swab (5583) isolates from the same patient.

‡ Serum 5586 used in this test.

Peebles(1). We did not repeat their filtration experiments, but were unable to recover fungi, or bacteria including pleuropneumonia-like organisms and leptospira from infected tissue cultures and presume, therefore, that the antigens that produced specific reactions in fluorescent antibody and complement-fixation tests were derived from measles virus. On the whole, antigen in infected tissue cultures was detected earlier by the fluorescent antibody method than by complement-fixation tests. Preliminary data suggest, however, that the latter are of value in differential diagnosis of exanthemata and encephalitides of unknown origin.

The intranuclear fluorescence that appeared after prolonged incubation of infected cultures looks like that observed by Leduc, Coons, and Connolly(12). They made indirect fluores-

cent antibody stains of sections of lymph nodes from rabbits immunized with diphtheria toxoid and found spots of antibody within nuclear shadows that closely resemble the intranuclear measles antigen we noted (cf. their Fig. 4 and 5, our Fig. 1 and 2). Whether the intranuclear measles antigen is associated with inclusion bodies demonstrable by Giemsa or other stains(1) is still to be determined. One of us has recently observed that poliomyelitis virus antigen appears later in the nuclei than in the cytoplasm of monkey kidney cells under circumstances quite similar to those in the present study(13).

Summary. Agents isolated in monkey kidney tissue cultures from 3 cases of measles were identified by immunologic tests with convalescent-phase measles serum. Measles antigen was detected in infected cells by the in-

TABLE II. Complement-Fixation Titers of Sera with Measles Agent Tissue Fluid Antigens.

Titer range*	Sera from cases of measles		Heterologous sera
	Days after onset†	<5	>5
<4	7	0	7
5-25	1	1	11
26-50	0	1	5
51-100		3	2
101-200	1		1
>200		4‡	

* Titers = Number of 50% units of complement fixed.

† Exact date of onset unknown in some cases; may refer to earliest symptoms or appearance of rash.

‡ Three additional sera from one patient taken 3 to 4½ months after onset fell in this group.

direct fluorescein-labeled antibody technic before specific complement-fixation antigen appeared in the fluid phase. Specific fluorescence due to the presence of measles antigen was noted in the nuclei as well as in the cytoplasm of some cells from infected cultures.

1. Enders, J. F., and Peebles, T. C., *PROC. SOC. EXP. BIOL. AND MED.*, 1954, v86, 277.

2. Coons, A. H., and Kaplan, M. H., *J. Exp. Med.*, 1950, v91, 1.

3. Weller, T. H., and Coons, A. H., *PROC. SOC. EXP. BIOL. AND MED.*, 1954, v86, 789.

4. Tompkins, V., and Macaulay, J. C., *J.A.M.A.*, 1955, v157, 711.

5. Youngner, J. S., *PROC. SOC. EXP. BIOL. AND MED.*, 1954, v85, 202.

6. Hazen, E. B., and Brown, R., *ibid.*, 1951, v76, 93.

7. Wadsworth, A. B., *Standard Methods of the Division of Laboratories and Research of the New York State Department of Health*, 3rd ed., Williams and Wilkins, Baltimore, Md., 1947, p. 226.

8. Buckley, S. M., Whitney, E., and Rapp, F., *PROC. SOC. EXP. BIOL. AND MED.*, in press.

9. Healy, G. M., Fisher, D. C., and Parker, R. C., *Canad. J. Biochem. and Physiol.*, 1954, v32, 327.

10. Rustigian, R., Johnston, P., and Reihart, H., *PROC. SOC. EXP. BIOL. AND MED.*, 1955, v88, 8.

11. Enders, J. F., personal communication, August 30, 1955.

12. Leduc, E. H., Coons, A. H., and Connolly, J. M., *J. Exp. Med.*, 1955, v102, 61.

13. Buckley, S. M., *Arch. ges. Virusforsch.*, in press.

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Chemotherapy of Experimental Schistosomiasis. IV. Oral Activity of Antimony Trichloride Antibiotic Complexes. (21958)

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(Introduced by Nathan B. Eddy.)

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1 - (Diethylaminoethylamino) - 4 - methylthioxanthone, Miracil D, was the first non-antimonial compound found to be of value for the oral treatment of schistosomiasis(1). In later studies, however, Miracil D proved to be toxic to man at therapeutic dosage levels(2,3). Other compounds which have been used with varying degrees of success in the oral treatment of schistosomiasis in man or experimental animals include antimony potassium tartrate, tartar emetic(4); sodium antimony III bis-pyrocatechol-2,4-disulfonate, Fuadin(5); various thioantimonials(6); and some symmetrical diaminodiphenoxyalkanes(7).

During the course of screening compounds in this laboratory, it was found that the antimony trichloride complexes of oxytetracycline, Terramycin; of chlortetracycline, Aureomycin; and of tetracycline were schistosomacidal in white mice. The present report includes a comparison of the oral activities of these complexes and of other antimony compounds.

Materials and methods. Samples of the 3 complexes were synthesized by Dr. Philip N. Gordon and were submitted for evaluation by Chas. Pfizer & Co., Inc. The dithiastibiole compound mentioned below was supplied

TABLE I. Toxicity and Activity of Antimony Compounds.

Compound	Acute oral LD ₅₀		Treatment results	
	Drug (mg/kg)	Sb equivalent (mg/kg)	No. mice	% reduction of worms by 10 oral doses total- ing 350 mg Sb/kg
Terramycin antimony trichloride	1450	174	35	53.5
Aureomycin antimony trichloride	1550	248	35	83.6
Tetracycline antimony trichloride	1110	187	24	91.0
Fuadin	>1200	>162	15	0
Tartar emetic	540	198	18	94.3
Antimony trichloride	510	272	14	81.2
4,5-Dihydro-2-hydroxy-4-methylol- 1,3,2-dithiastibiole	>1575	>732	9	0

through the courtesy of Abbott Laboratories. The drugs were given orally to white Swiss mice. In addition, the complexes were tested by the subcutaneous and intraperitoneal routes. After the compounds were found to be schistosomacidal by the preliminary method of screening described by Luttermoser(8), further evaluation of their activities was carried out as follows. Commencing on the 35th day after exposure to about 300 *Schistosoma mansoni* cercariae, mice in groups of 5 or 6 each were treated orally twice daily with the same dose of antimony in the form of one of the different compounds for a period of 5 days. Two to 3 weeks after treatment, the animals were killed and the schistosomes living in the portal system were perfused out by the technic of Yolles *et al.*(9) and counted. Small pieces (1 cm²) of the livers of the treated mice were pressed between glass slides and examined for dead worms trapped in lesions. Unless dead worms were found, no reduction in worm infection was claimed. The worms living in the untreated mice were likewise isolated and counted by the same procedure. The percentage of worms remaining alive in the treated groups was inferred from the total number of living worms recovered from the treated mice relative to the number obtained from the untreated mice. From these data the percentage reduction in infection resulting from treatment was calculated. The LD₅₀ values were determined on mice by the method of Litchfield and Wilcoxon(10).

Results. Table I summarizes the data on the activity and toxicity of the various compounds. The minimum oral dose of the Terramycin antimony trichloride complex which

cleared the mice of more than one-half of their parasites was about 300 mg/kg (equivalent to about 35 mg antimony/kg) of body weight, while the oral LD₅₀ of this compound for uninfected mice was about 1450 mg/kg(11). Fuadin, one of the drugs of reference, was not schistosomacidal at oral doses as high as 425 mg/kg. The curative results obtained with the first complex given orally at a level equivalent to 35 mg antimony/kg twice daily for 5 days were compared with those obtained by giving the same oral dose of antimony in the form of tartar emetic, antimony trichloride, and 4,5-dihydro-2-hydroxy-4-methylol-1,3,2-dithiastibiole. The Terramycin complex was more active than the last-named compound, which was reported by Schubert(6) to be among the most active schistosomacidal compounds when fed to mice at higher levels than used in this study. In comparison with tartar emetic and with antimony trichloride, the Terramycin antimony complex demonstrated no advantage in activity at this dosage. When Terramycin and antimony trichloride in quantities equivalent to the dosage of the complex were given separately but one following the other, reduction of live parasites equal to that obtained by administration of the Terramycin antimony complex was observed. Oral regimens of Terramycin, Aureomycin, or tetracycline alone were not schistosomacidal at oral doses comparable to the amount of these antibiotics in the complexes. Preliminary comparison of the activities and toxicities of the Terramycin antimony complex with the Aureomycin and tetracycline complexes (Table I) indicates that the last 2 are more active than the first but have similar toxicities.

Thus the apparent therapeutic advantage of the Aureomycin antimony complex or the tetracycline antimony complex over the Terramycin antimony complex is questionable. The 3 antibiotic antimony complexes were active following subcutaneous or intraperitoneal injection, but were toxic at doses of 50 mg/kg and higher.

Discussion. It appears probable that the schistosomacidal activity and toxicity of the 3 antibiotic complexes result almost entirely from their antimony content, and that variations in the efficacy of these compounds compared with the other antimony-containing preparations mentioned arise from differences in solubility, absorption, and dissociation. Such variations in activity further emphasize the need for additional investigation of organic antimonial compounds which are schistosomacidal upon oral administration.

Summary. The antimony trichloride complexes of Terramycin, Aureomycin, and tetracycline were active orally against *S. mansoni*

infections in white Swiss mice. These compounds were no more active than tartar emetic or antimony trichloride.

1. Kikuth, W., Gonnert, R., and Mauss, H., *Naturwiss.*, 1946, v33, 253.
2. Halawani, A., *J. Roy. Egyptian M. A.*, 1949, v32, 29.
3. da Silva, J. R., *Rev. brasil. med.*, 1953, v9, 577.
4. Walker, J., *Ann. Soc. belge de méd. trop.*, 1928, v8, 273.
5. El Ayadi, S. M., *J. Roy. Egyptian M. A.*, 1947, v30, 562.
6. Schubert, M., *Am. J. Trop. Med.*, 1948, v30, 525.
7. Raison, C. G., and Standen, O. D., *Brit. J. Pharmacol.*, 1955, v10, 191.
8. Luttermoser, G. W., *J. Parasitol.*, 1954, v40, 130.
9. Yolles, T. K., Moore, D. V., DeGiusti, D. L., Ripsom, C. A., and Meleney, H. E., *ibid.*, 1947, v33, 419.
10. Litchfield, J. T., Jr., and Wilcoxon, F., *J. Pharmacol. and Exp. Therap.*, 1949, v96, 99.
11. Personal communication from Dr. J. E. Lynch, Pfizer Therapeutic Institute.

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Effect of Liver Damage and Nephrectomy on Convulsant Potency of Pentylene-tetrazol (Metrazol)* (21959)

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There is lack of agreement on the fate and excretion of pentylene-tetrazol (Metrazol) in laboratory animals and man. On the basis of data obtained by *chemical procedures*, Tatum and Kozelka(1) concluded that Metrazol is detoxified by the liver in dogs and man, whereas Esser and Kuehn(2) reported that the major portion of the drug is excreted by the kidney in man. Recent studies in our laboratory(3) with radioactive Metrazol indicate that most of a given dose of the drug in rats is excreted by the kidney in an altered form. On the basis of data obtained by *biological*

procedures, Dille and Seeberg(4) concluded that Metrazol is degraded by the liver in cats, whereas Voss(5) reported that the drug is largely excreted by the kidney in rats. In view of these conflicting reports, it was thought important to make a systematic study of the effect of liver damage and of nephrectomy on the convulsant activity of this agent. It was anticipated that such a study would contribute information on the relative role of the liver and the kidney in the detoxification and excretion of Metrazol. The results obtained provide the basis for this report.

Methods. Adult male albino mice obtained from the Carworth Farms (CF #1) and adult male albino rats obtained from the Holtzman-Rolfsmeyer Farms were used as experimental

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animals. They were maintained on Purina Laboratory Chow and allowed free access to food and water except during the actual experimental procedure. The convulsant potency of *intravenously* administered Metrazol[†] was determined in control, liver-damaged, and nephrectomized mice; the convulsant potency of *subcutaneously* administered Metrazol was determined in control, liver-damaged, nephrectomized, and sham-operated mice and rats. In the determination of the convulsant potency, groups of at least 8 animals were given various doses of the drug until at least 3 points were established between the dose required to convulse 16% of animals and that to convulse 84% of animals. The results obtained were then plotted on logarithmic probability paper and a regression line visually fitted to the plotted points. From this plot of the data, the dose of Metrazol which was convulsant to 50% of animals (CD_{50}) was determined and the 95% confidence limits were calculated by the method of Litchfield and Wilcoxon(6). Intravenously, the drug (0.5% aqueous solution) was injected rapidly into the dorsal tail vein of the mouse; the endpoint employed was either a minimal seizure (any overt clonic activity which persisted for at least 5 seconds), or a full maximal seizure as indicated by the presence of the hindleg tonic extensor component(7). Subcutaneously, the drug (0.5% aqueous solution in mice, 5.0% in rats) was injected into a loose fold of skin over the neck; the endpoint employed was the occurrence of a minimal seizure, as described above, within one hour after the injection. Liver damage in mice was produced by a modification of the method of Stowell and Lee (8), *i.e.*, by the oral administration of 4 ml/kg of a 40% (w/v) solution of carbon tetrachloride (CCl_4) in olive oil. Maximum hepatic damage occurs about 100 hours after such administration and therefore Metrazol was tested at this time. Liver damage in rats was induced by a single subcutaneous injection of 2 ml/kg of a 50% solution (w/v) of CCl_4 in peanut oil. Histological studies of livers taken from rats treated in this manner

TABLE I. Effects of Liver Damage and of Nephrectomy in Mice on Convulsant Potency of Intravenously Administered Pentyletmetrazol (Metrazol).

Exp. procedure	Convulsant potency (CD_{50}), mg/kg (intrav.)	
	Max seizures	Min seizures
Control	29.0 (27.1-31.0)	21.0 (19.3-22.9)
Liver damage	29.0 (26.8-31.4)	19.5 (17.4-21.8)
Nephrectomy	28.5 (27.0-30.1)	24.5 (22.7-26.5)

Values in parentheses represent 95% confidence limits.

have shown that maximum central and mid-zonal necrosis occurred approximately 48 hours after the injection of CCl_4 ; accordingly, Metrazol was tested at this time. Bilateral nephrectomy was performed in both species by the retroperitoneal approach while the animals were under light ether anesthesia. In order to keep the effect of accumulated metabolic products relatively constant, Metrazol was tested 6 and 12 hours after nephrectomy in mice and rats, respectively. Sham operation was performed by the same technic as for nephrectomy, except that the kidneys were not disturbed.

Results. The effects of liver damage and of nephrectomy in mice on the convulsant potency of intravenously administered pentyletmetrazol (Metrazol) are shown in Table I. The CD_{50} s for *intravenous* Metrazol for *maximal* seizures determined in liver-damaged mice and nephrectomized mice were not significantly different from the CD_{50} determined in control animals. On the other hand, the CD_{50} for *intravenous* Metrazol for *minimal* seizures, a more sensitive test, tended to be lower in liver-damaged mice and higher in nephrectomized mice. In view of these observations, it was decided to determine the CD_{50} of Metrazol administered by a route which would provide a longer time interval before the onset of convulsions than that provided by the intravenous route. It was anticipated that the longer sojourn of Metrazol in the body would induce more marked differences in the CD_{50} s determined in liver-damaged and nephrectomized animals, and thus delineate the relative

[†] Kindly supplied by Dr. C. L. Moench, Bilhuber-Knoll Corp.

TABLE 11. Effect of Liver Damage and of Nephrectomy in Mice and Rats on Convulsant Potency of Subcutaneously Administered Pentylentetrazol (Metrazol).

Exp. procedure	Convulsive potency (CD_{50}), mg/kg	
	s.c. route—min seizures	
	Mice	Rats
Control	59.0 (55.2-63.1)	47.0 (43.9-50.3)
Liver damage	46.5 (43.3-50.0)	39.5 (36.4-42.9)
Nephrectomy	66.0 (59.5-73.3)	45.5 (41.9-49.6)
Sham operation	65.0 (59.6-70.9)	42.0 (38.7-45.6)

Values in parentheses represent 95% confidence limits.

importance of these organs in the fate and excretion of the drug. Accordingly, Metrazol was injected *subcutaneously* in mice and rats and the CD_{50} for *minimal* seizures determined. The results obtained are summarized in Table II. The CD_{50} s for subcutaneous Metrazol are significantly lower in liver-damaged mice (21%) and rats (18%) than the control CD_{50} s. In sharp contrast, the CD_{50} s for subcutaneous Metrazol in nephrectomized mice and rats were not significantly different from the CD_{50} s in control or sham-operated animals. These data suggest that the liver plays the major role in the metabolic degradation of Metrazol in both species.

Discussion. The fact that CCl_4 -induced liver damage and nephrectomy in mice had no significant effect on the intravenous CD_{50} of Metrazol is easily explained. The rapid intravenous injection of Metrazol elicits seizures so quickly that the organs concerned with the detoxification and excretion of this drug have only seconds in which to exert an effect. Hence, any influence which the liver or the kidney may exert is largely circumvented.

Liver damage lowered the CD_{50} for subcutaneously administered Metrazol by 21% in mice and 18% in rats. This suggests that the liver is important for the degradation of this drug. Additional evidence in support of this conclusion is the fact that fatalities after Metrazol were more frequent in liver-damaged than in normal mice. For example, in the dose range of 40 to 47 mg/kg, subcutaneous

Metrazol was fatal to 37.5% of liver-damaged mice, whereas all normal mice survived the drug even in the higher range of 50 to 66 mg/kg. These observations are in agreement with the evidence that Metrazol is converted into an inactive product and that the site of this conversion is the liver(9).

Since there was no significant difference in the CD_{50} s of subcutaneously administered Metrazol determined in control, nephrectomized, and sham-operated mice and rats, it would appear that, within the time interval required to absorb the drug after subcutaneous injection, no appreciable amount of Metrazol is excreted by the kidney. This interpretation is in agreement with the observation of Esplin *et al.*(3) that less than 1% of intravenously administered radioactive (C^{14}) Metrazol is excreted by the rat kidney as an inactive product during the first 30 minutes, and that a total of 75% is excreted in an inactive form over a 48-hour period. Thus, the kidney appears to play a relatively minor role in the excretion of Metrazol or of an active conversion product.

Summary. The convulsant potency of intravenously administered Metrazol for maximal and minimal seizures was determined in control, liver-damaged, and nephrectomized mice. In addition, the convulsant potency of subcutaneously administered Metrazol for minimal seizures was determined in control, liver-damaged, nephrectomized, and sham-operated mice and rats. The results obtained were analyzed to determine the relative importance of the liver and the kidney in the fate and excretion of the drug. Neither liver damage nor nephrectomy had any significant effect on the potency of intravenously administered Metrazol for maximal or minimal seizures. Liver damage significantly increased the potency of subcutaneously administered Metrazol for minimal seizures; on the other hand, bilateral nephrectomy and sham operation had no significant effect on the potency of this drug. The data indicate that, in both mice and rats, the liver is the principal organ for the conversion of Metrazol into products devoid of convulsant activity, and that, within the time required for the complete absorption of subcutaneously injected Metra-

zol; the kidney plays no important role in the metabolic alteration or excretion of this compound.

1. Tatum, H. J., and Kozelka, F. L., *J. Pharmacol. and Exp. Therap.*, 1941, v72, 284.
2. Esser, A., and Kuehn, A., *Deutsche Z. ges. gerichtl. Med.*, 1933, v21, 474.
3. Esplin, D. W., Woodbury, D. M., and Goodman, L. S., *Fed. Proc.*, 1954, v13, 352.
4. Dille, J. M., and Seeberg, V. P., *PROC. SOC. EXP. BIOL. AND MED.*, 1940, v44, 624.

5. Voss, J., *Arch. f. exp. Path. u. Pharmacol.*, 1926, v118, 259.
6. Litchfield, J. T., Jr., and Wilcoxon, F., *J. Pharmacol. and Exp. Therap.*, 1949, v96, 99.
7. Goodman, L. S., Grewal, M. Singh, Brown, W. C., and Swinyard, E. A., *ibid.*, 1953, v108, 168.
8. Stowell, R. E., and Lee, C. S., *Arch. Path.*, 1950, v50, 519.
9. Esplin, D. W., unpublished observations, 1954.

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Biological Method for Determination of Glucocorticoid Activity of Crystalline Compounds and Urine Extracts.* (21960)

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During the past decade, several biological methods for assay of compounds exhibiting glucocorticoid activity have been established. These technics have been applied to the assay of urine extracts to measure excretion levels of adrenal cortical hormone by the human. The methods of Reinecke and Kendall(1), Olson, Jacobs, Richert, Thayer, Kopp, and Wade(2), and Pabst, Sheppard, and Kuizenga (3), are based on capacity of glucocorticoids to deposit glycogen in livers of adrenalectomized rats after glycogen stores have been depleted by fasting. These methods use male rats weighing 140 to 180 g and injections of test material are made at regular intervals throughout the assay. The methods are reasonably specific for assay of glucocorticoids and reproducible with a fairly high degree of precision. The disadvantage of the methods lies in their comparative lack of sensitivity. Lack of sensitivity was apparently overcome by using the mouse as assay animal. The method of Venning, Kazmin, and Bell(4) measures deposition of glycogen in depleted

livers of fasting adrenalectomized mice, while those of Eggleston, Johnston and Dobriner (5), and of Dorfman, Ross and Shipley(6) measure capacity of adrenal corticoids to prevent loss of hepatic glycogen during fasting. Our experience with the method of Venning, Kazmin and Bell demonstrated the assay to be unreliable with strains of mice available to us. Nissim(7), who conducted a critical study of various methods, believes that precision obtained by Venning *et al.* is dependent upon selection and careful control of a particularly suitable strain of mice bred in their own laboratories.

Existing methods were not suitable for our needs. The methods using young adult rats were not sensitive enough for assay of urine extracts, and uneconomical from the standpoint of animal cost and amount of test material necessary for assay. The mouse methods offered economy both in animals and test materials, but the strains of mice generally available did not lend precision to the assay. It therefore became desirable to develop a method based on satisfactory features of other methods. The method we devised is presented in this communication.

Materials and methods. The animals were 21-day-old male rats of the Holtzman strain weighing 50 to 55 g. These animals were

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placed in a cage, given Rockland Rat Diet and tap water *ad libitum*, to become accustomed to laboratory conditions for a period of 2 days. After this the animals were bilaterally adrenalectomized and returned to the cage. Animals had access to a solution of 5% cerelese in 0.9% NaCl as drinking water until the following morning, then the solution was replaced with one containing 0.9% NaCl only. A good supply of food was available at all times until the evening of the second post-operative day. The animals were then deprived of food. On the morning of the third post-operative day, saline drinking solution was removed, the test compounds injected subcutaneously, and each animal placed in an individual quart Mason jar containing about 2 inches of sawdust. The jars were covered with sealer rings fitted with hardware cloth mesh. It is extremely important that rats be isolated from one another at this point. Occasionally an animal of the control group, or one given an inactive preparation, becomes hypoglycemic and subject to convulsions. Such animals tend to fight with other rats if they are caged together. Test materials were administered in 0.1 cc of corn oil per rat. Cortisone was first dissolved in acetone, an aliquot then transferred to corn oil so that the desired dosage was contained in 0.1 cc, and acetone evaporated by vacuum. Six hours after injection of test material, the animals were individually weighed, sacrificed by cervical dislocation, the entire liver quickly removed and transferred to a hot 30% solution of KOH for digestion. A 50 cc pyrex, round-bottom centrifuge tube containing 10 cc of KOH solution proved to be very satisfactory. Small funnels were placed in top of the tubes to serve as condensers and the tubes heated in boiling water bath just previous to autopsy. Digestion process was complete in 30 or 40 minutes, after which the glycogen was precipitated by adding absolute ethyl alcohol (about 1.2 times the volume of the digest) while the digests were still hot. The digests were then brought to a boil, cooled in a cold water bath, again brought to a boil and re-cooled as suggested by Pabst, Sheppard and Kuizenga(3). The KOH-liver digests were placed in refrigerator for at least 2 hours

to insure more complete precipitation and coagulation of the glycogen. If necessary, the liver digests may be refrigerated over night. After this, the samples were centrifuged at 2500 rpm for 10 minutes, the supernatant decanted and discarded, and the tubes inverted on absorbent paper and allowed to drain. The precipitated glycogen was then washed with a small amount of absolute alcohol, centrifuged, and again allowed to drain. Glycogen was hydrolyzed by adding 6 cc of 0.6N HCl and placing the tubes in an autoclave at 15 lb pressure for 20 minutes. After cooling to room temperature the hydrolysates were neutralized with 10% NaOH using Phenol Red as an indicator. The solutions were centrifuged, and glucose determined by the method of Reinecke(8). The values obtained were then expressed as glucose per 100 g, body weight per rat.

Results. *Determination of time for autopsy.* In the initial phases it was important to determine the most favorable time at which animals should be sacrificed after injection of cortisone, which was to be employed as the standard. Groups of animals were killed at 2, 4, 6, 8 and 10 hours after injection and amount of liver glycogen deposited was determined. The cortisone in this experiment was 100 μ g, a dose which preliminary study indicated would give a good response and yet be somewhat less than maximal. The results of this study are shown in Table I. During the first 2-hour period after injection of cortisone there was no indication of glycogen deposition over and above the level found in oil-treated control animals. At 4 hours the animals had deposited a highly significant amount of gly-

TABLE I. Amount of Liver Glycogen Deposited at 2-Hr Intervals after 100 μ g Cortisone Injection.

Interval, hr	No. animals	Glycogen,* avg \pm S.E., mg	Increase, mg	"t"
0				
2	7	.66 \pm .046		
4	9	10.14 \pm .58	9.473	14.013+
6	8	20.25 \pm 1.52	10.11	6.46 +
8	9	29.02 \pm 1.46	8.77	4.16 +
10	7	22.76 \pm 5.25	-6.26	
Oil controls at 6 hr	6	.68 \pm .074		

* Glycogen expressed as glucose/100 g body wt.
+ P = <0.01.

TABLE II. Glycogen Deposition in Response to Various Doses of Cortisone.

Dose cortisone, μ g	No. animals	Glycogen, mg Avg	S.E.	C, %	"t"
0	25	.68	.074	54	
10	12	2.52	.329	45	7.99†
30	13	9.69	.925	34	7.1 †
50	17	13.8	1.08	32	2.79†
100	18	22.64	1.07	20	4.9 †
200	18	28.67	2.24	33	2.43†
300	19	39.35	1.74	19	3.75†

* Coefficient of variation; expresses stand. dev. as % of mean response. Avg "C" for all cortisone doses is 30.5%.

† $P < .05$.

‡ $P < .01$.

Glycogen expressed as mg glucose/100 g body wt.

cogen. Expressed as mg glucose/100 g rat weight, a level of 10.14 mg was found. At 6 hours and again at 8 hours post-injection, highly significant increases in liver glycogen were noted. During the 2-hour period from 6 to 8 hours, deposition of glycogen was somewhat less than that observed during the preceding 2-hour period, and at 10 hours post-injection, the peak glycogen deposition in response to a single injection of cortisone had been passed and glycogen was again being mobilized. It was then decided that 6 hours post-injection was the optimum time for sacrificing the animals. During the period between 2 and 8 hours post-injection, the deposition of liver glycogen progressed as a linear function of time. After 8 hours the concentration of glycogen in the liver decreased. It was considered preferable to select a time during which glycogen was being deposited at a steady rate and before the peak was reached.

Development of a dose-response curve. Cortisone was employed as the standard hormone in development of the assay method, and the data in Table II show that a satisfactory dose-response relationship exists over a relatively large range of doses. Highly significant differences in glycogen deposition were obtained with 20 μ g increments in dosage of cortisone in the lower dosage range. A measurable and highly significant response was obtained with as little as 10 μ g of cortisone. These results indicate that the assay is much more sensitive than any of the existing

methods utilizing the 140 to 180 g rat. In the method suggested by Pabst, *et al.*, a dose of 350 μ g of cortisone was necessary to deposit a significant amount of glycogen in the liver, and dose increments of 30 μ g were necessary to elicit significant increases in liver glycogen. The coefficient of variation(9) has been calculated for each dosage and the average coefficient of variation for cortisone is shown in Table II. Averages for the methods of Olson, *et al.* and Pabst, *et al.*, as calculated by Nissim(7), are 29.5% and 23.25% respectively. The average reported here is 30.5% indicating only slightly higher variation in these animals. The method of Venning (using her strain of mice) is perhaps more sensitive to smaller changes in dosage of cortisone than the method suggested here. She has shown a significant change in glycogen deposition with as small an increment as 10 μ g of cortisone, but does not report any liver glycogen values for smaller doses. The 10 μ g dose is also in the lower limit for a significant response in our method.

The data in Table II have been used to plot the dose-response curve found in Fig. 1. The mean values of glucose per 100 g weight were plotted against the log.-dose of cortisone and the curve obtained indicated a straight line relationship. From these values the slope,

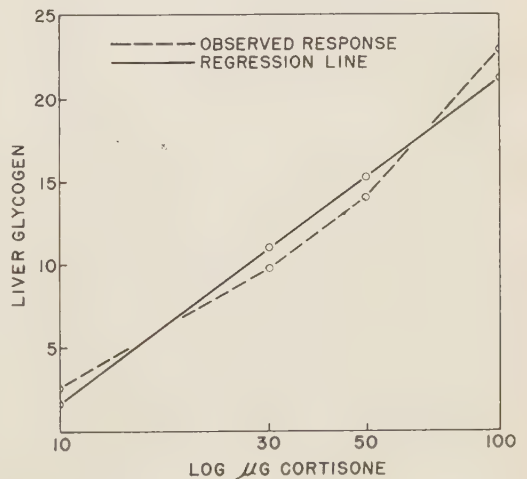


FIG. 1. The mean values for liver glycogen expressed as mg of glucose per 100 g body wt, plotted against the log-dose of cortisone.

"b", which was calculated according to the formula $\frac{\sum xy}{\sum x^2}$ as discussed by Snedecor(9), was found to be 19.559. The regression line was then calculated according to the formula $\hat{Y} = \bar{y} + b(X - \bar{x})$. The index of precision was calculated according to the formula $\lambda = s/b$ wherein "s" is equal to the average standard deviation about the curve and "b" is the slope of the regression line. According to Emmens(10) the value of λ is a guide to the precision of the assay; a minimal value for λ indicates a steep regression line in relation to magnitude of variance.

It is evident from Table II that as the dose increases above 100 μ g of cortisone, there is a sharp rise in the standard error of the mean. Precision indices were calculated using the data from doses a) 0 to 300 μ g of cortisone; b) 0 to 200 μ g of cortisone; and c) 0 to 100 μ g of cortisone. The indices were found to be 0.225, 0.273, and 0.183, respectively. Accepting the fact that for a precise bioassay the value for lambda should be minimal, it is apparent that the range of doses from 0 to 100 μ g of cortisone gives the most precise information.

Assay of glucocorticoids in urine. The primary purpose for the development of this assay was to establish a method by which levels of glucocorticoid material could be measured in human urine. Chloroform extracts of urine were prepared according to Mason's modification(11) of an original method for chemical determination of corticoids by Daughaday, Jaffe and Williams(12). Some difficulty was encountered using the crude chloroform extracts of normal urines. Attempts to show a dose-response relationship with normal urine were unsuccessful at the outset. Response to increasing doses of urine extracts was not a linear increase in liver glycogen. In many cases the results showed a decrease in amount of glycogen as the dose of urine extract was increased. This pattern of results suggested that either there was some active principle in urine extracts that was inhibiting glucocorticoid activity, or that there was inactive material, poorly soluble in the volume of oil used, that was interfering with absorption of

TABLE III. Effect of Benzene-Water Partition of Urine Extracts on Glycogen Deposition.

Extract	Dose equivalent, hr	Glycogen crude extract, mg	Glycogen benz. fract., mg	Glycogen HOH fract., mg
A	3	.646		1.45
	6	1.27	.706	2.15
B	2	30.28	1.62	32.14

Glycogen expressed as mg glucose/100 g body wt.

the glucocorticoids. Many of the urine extracts possessed large amounts of color, all of which appeared to be readily soluble in any of the non-polar solvents. In view of the behavior of crystalline steroids in a benzene-water partition system, as shown by Talbot, Saltzman, Wixom and Wolfe(13), it was believed that this treatment might be beneficial in purifying urine extracts. Talbot, *et al.* found that if the active corticoid compounds were dissolved in benzene and partitioned with water, the only compound that remained in the benzene to any extent was desoxycorticosterone. The more highly oxygenated steroids preferentially moved into the water phase. This procedure was applied to urine extracts and it was found that the pigmented material remained almost entirely in the benzene fraction and that essentially all materials active in liver glycogen deposition moved into the water phase. The experimental data are presented in Table III.

Benzene-water partitioning considerably reduced the amount of solid material in urine extracts, giving a cleaner preparation that could be dissolved in a small volume of oil for injection. There is some indication that higher levels of glycogen deposition are obtained with the same hourly equivalents of urine extract after a benzene-water partition.

The data obtained from the assay of urine extracts from normal male individuals is shown in Table IV. A dose-response relationship is indicated which shows a highly significant change in glycogen deposition between 200 and 400 ml urine equivalent doses, and a significant change between 400 and 600 ml dose levels. These data are expressed graphically in Fig. 2 together with the standard curve for cortisone for comparison. Re-

TABLE IV. Glycogen Deposition in Response to Doses of Urine Extract from Normal Individuals.

Dose equivalent, ml	No. animals	Glycogen,* mg		"t"	Cortisone† equivalent, μ g	Cortisone equiv./l, μ g
		Avg	S.E.			
200	6	1.24	.37		9.6	48.0
400	5	6.31	1.18	4.43§	17.5	43.75
600	6	9.09	.51	2.30‡	24.0	40.0

* Glycogen expressed as glucose/100 g body wt.

‡ $P = <.05$.§ $P = <.01$.

† Determined from stand. curve; Fig. 1.

gression lines for cortisone and for urine extract, the slope of which was 16.553, were tested statistically to see if they deviated significantly from parallel. The value "t" was calculated according to the formula:

$$t = \frac{b_1 - b_2}{\sqrt{\sigma_{d^2}}}$$

The value " $b_1 - b_2$ " is equal to

the difference between slopes and the quantity $\sqrt{\sigma_{d^2}}$ is the standard error of the difference between regression coefficients(14). The value for "t" was 1.633 which showed the two regression lines did not deviate from parallel significantly. Therefore, it can be assumed that urine extracts may be assayed in terms of cortisone. When calculated as μ g equivalents of cortisone excreted per liter of urine, as shown in Table IV, it is clear that fairly close agreement in results exists between the dose levels administered.

A comparison of the values for corticoid ex-

cretion obtained by this method with determinations made by other investigators also show close agreement. This method shows an average excretion of 44 μ g cortisone/l of pooled normal male urine. Schneider(15) has obtained a value of 53.1 μ g/l, Zaffaroni (16) reports a range of 20 to 40 μ g/l, and Venning(17) reports values of 40 to 80 μ g/l of urine of normal individuals. Determinations of Schneider and of Zaffaroni were by means of paper chromatography and Venning's values by biological assay.

Validity of the assay procedure for measurement of variations in corticoid excretion is demonstrated by an experiment wherein a patient was given ACTH intravenously for periods of 3 days interspersed with 3-day control periods. Urine was collected, extracted, and assayed for glucocorticoids by the bioassay method reported here. Table V shows glucocorticoid, and the formaldehydogenic corticoid excretion in response to ACTH stimulation. Results showing an increase in excretion of glucocorticoids during ACTH administration, indicate that this method measures secretion products of the adrenal cortex which influence carbohydrate metabolism, and that the method is sufficiently sensitive to detect fluctuations in excretion of glycogenic steroids in urine.

In Table V the results of both assays are expressed as μ g of cortisone excreted per 24 hours. It can be seen that by chemical assay the excretion level is considerably higher than that shown by biological determination. This indicates that chemical determination is measuring formaldehydogenic compounds which do not have glycogenic activity. In the calculation of corticoid excretion the assumption is made that 1 mole of formaldehyde is derived from 1 mole of formaldehydogenic ster-

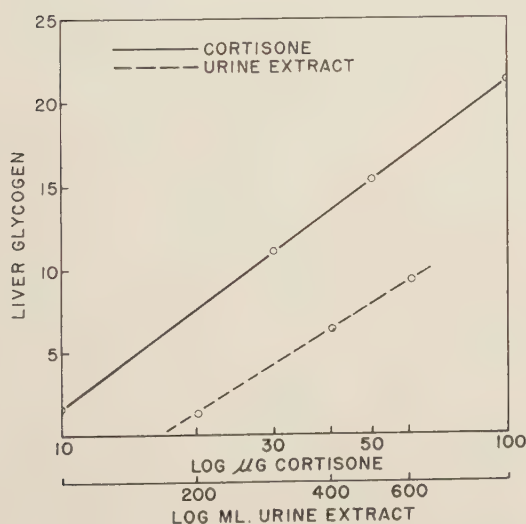


FIG. 2. Regression line for liver glycogen response to urine extracts compared with the regression line for response to cortisone.

TABLE V. Daily Corticoid Excretion in Urine during ACTH Stimulation.

Treatment*	Glucocorticoids,	FGS,†
	μg equiv. “E”/24 hr	μg “E”/24 hr
Control	91.2	931.
5 mg ACTH	2380.	3870.
Control	120.	1167.
10 mg ACTH	1298.	3794.
Control	266.	675.
25 mg ACTH	1440.	4125.

* Consecutive 3-day periods.

† Formaldehydogenic steroids.

oid. This method of calculation does not make allowance for differences in glycogen-depositing activity of various formaldehydogenic steroids. It is well known that the formaldehydogenic method measures biologically inactive metabolites of glycogenic steroids as well as active forms. Since the isolation of pregnance-3, 17, 21-triol 11, 20-dione (tetrahydro E) in 1951 by Schneider(15), much investigation into excretion of corticoid metabolites has been conducted.

The introduction of -glucuronidase by Kinsella, Doisy and Glick(18) for hydrolysis of urinary steroids greatly increased the yield of chemically detectable corticoids. Interesting studies on urine and blood levels of corticoids and their metabolites have recently been reported by Baggett, Kuisella and Doisy(19) and Silber and Porter(20). These investigators have also found increased yields of urinary corticoids by enzymatic hydrolysis and, in addition, Silber and Porter showed that the main corticoid in blood is hydrocortisone (Compound F) while in urine it is tetrahydro F, 94% of which is excreted as a glucuronide. Studies by Fukushima, Leeds, Bradlow, Kritchevsky, Stokem and Gallagher, (21) have disclosed 4 new metabolites excreted in urine following ACTH administration. These compounds, designated by the authors as “cortol, -cortol, cortolone, and -cortolone,” are not reducing substances and are negative to the Porter-Silber reaction, however, they do yield formaldehyde on periodic acid oxidation.

These increased yields of urinary corticoids and newly discovered metabolites tend to further the understanding of adrenal function and metabolism of cortical secretions to a de-

gree that could not have been arrived at by means of a biological assay alone. It has, however, been shown by Venning(22), Lobotsky, Hannye and Lloyd(23), and Chart(24), that for the routine bioassay of urinary corticoids the hydrolysis of urine with -glucuronidase does not add greatly to the information obtainable by acid hydrolysis. The great increase in yield of chemically detectable corticoids is not reflected by a comparable increase of activity in biological assay procedures. This fact does not negate the usefulness of bioassay procedures or of the enzymatic hydrolysis of urine for determination of corticoid excretion in urine. It is of importance to establish the relative activity of newly isolated metabolites and to quantitate the potency of new synthetic steroids as they become available.

We believe that this bioassay procedure can function as a valuable adjunct to the chemical methods for the evaluation of corticoid excretion as well as being an accurate, simple and economical method for determination of glycogen-depositing activity of crystalline compounds.

Summary. A practical bioassay technic for glucocorticoids is proposed, using the adrenalectomized, 21-day-old male rat as test animal. The method offers the accuracy and precision shown by the use of the young adult rat, and the sensitivity and economy observed in methods using the laboratory mouse. Dose-response curves have been established for cortisone and for the assay of urine extracts. The regression lines for the two substances are parallel, indicating the method is valid for measurement of glucocorticoid excretion. It is suggested that this bioassay method may serve as a valuable adjunct to the chemical methods for the assay of urinary corticoids.

1. Reinecke, Roger M., and Kendall, Edward C., *Endocrinology*, 1942, v31, 573.
2. Olson, R. E., Jacobs, F. A., Richert, D., Thayer, S. A., Kopp, L. J., and Wade, N. J., *ibid.*, 1944, v35, 430.
3. Pabst, M. L., Sheppard, R., and Kuizenga, M. H., *ibid.*, 1947, v41, 55.
4. Venning, E. H., Kazmin, V. E., and Bell, J. C., *ibid.*, 1946, v38, 79.
5. Eggleston, N. M., Johnston, B. J., and Dobriner,

- K., *ibid.*, 1946, v38, 197.
6. Dorfman, R. I., Ross, E., and Shipley, R. A., *ibid.*, 1946, v38, 178.
7. Nissim, J. A., *ibid.*, 1953, v52, 611.
8. Reinecke, Roger M., *J. Biol. Chem.*, 1942, v143, 351.
9. Snedecor, George W., *Statistical Methods*, 4th ed., Iowa State College Press, Ames, Iowa, 1946.
10. Emmens, C. W., *Hormone Assay*, Academic Press, Inc., New York, 1950.
11. Mason, H. L., personal communication.
12. Daughaday, W. H., Jaffe, H., and Williams, R. H., *J. Clin. Endocrinol.*, 1948, v8, 166.
13. Talbot, N. B., Saltzman, A. H., Wixom, R. L., and Wolfe, J. K., *J. Biol. Chem.*, 1945, v160, 535.
14. Fisher, R. A., *Statistical Methods for Research Workers*, G. E. Stechert & Co., New York, 1941.
15. Schneider, John J., *J. Biol. Chem.*, 1952, v194, 337.
16. Zaffaroni, A. Burton, R. B., and Keutmann, E. H., *Science*, 1950, v6, 11.
17. Venning, E. H., and Kazmin, V. E., *Endocrinology*, 1946, v39, 131.
18. Kinsella, R. A., Jr., Doisy, R. J., and Glick, J. H., Jr., *Fed. Proc.*, 1950, v9, 190.
19. Bagget, Billy, Kinsella, R. A., and Doisy, E. M., *J. Biol. Chem.*, 1953, v203, 1013.
20. Silber, R. H., and Porter, C. C., *ibid.*, 1954, v210, 923.
21. Fukushima, D. K., Leeds, N. S., Bradlow, H. L., Kritchevsky, T. H., Stokem, M. B., and Gallagher, T. F., *ibid.*, 1955, v212, 449.
22. Venning, E. H., *Fed. Proc.*, 1952, v11, 302.
23. Lobotsky, J., Hannye, J. B., and Lloyd, C. W., Abstract No. 124, Program, Endocrine Society, Atlantic City, June, 1955.
24. Chart, J. J., Ph. D. Thesis, University of Wisconsin, 1954.

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Colorimetric Study of Absorption and Excretion of 4-(p-Dimethylaminostyryl) Quinoline Methiodide and Related Compounds.* (21961)

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Since 4-(p-dimethylaminostyryl) quinoline methiodide(1) and several related dyes, when administered in the diet, have been found to cause regression of Lymphoma 8 tumors in rats(2-4), the fate of the unchanged dye was studied colorimetrically. The results of this study have led to finding a compound that appears to be unusually active in bringing about regression of Lymphoma 8(3,4).

Methods. The compounds used were prepared in the chemical laboratories of Carson-Newman College by Wilfred Lyke and Joan Wilson, with the financial assistance of a Frederick Gardner Cottrell Grant from the Research Corporation and a grant from the Medical Research Foundation: 4-(p-Dimethylaminostyryl) quinoline methiodide (4M2M). 4-(p-Dimethylaminostyryl) quinoline methochloride (4M2MCl). 2-(p-Diethylaminostyryl) quinoline ethiodide (2E2E). 2-

(p-Dimethylaminostyryl) pyridine methiodide (2M2PM)[‡], and 2-(p-Dimethylaminostyryl) pyridine methiodide (2M2NPM). The usual method of administering the dyes was to mix them with powdered food of animals, by grinding in a ball mill 8 hours or longer. After offering dyed food for 2 days the animals were given untreated food *ad libitum*, beginning the evening of second day. In a few instances a solution of a dye in a 0.9% NaCl or 5% glucose was administered by a stomach tube or was injected subcutaneously in the flank or intravenously via a tail vein. Urine and feces were collected separately, by conventional apparatus, until 2 days after last dose of dye was administered. The dye was

[‡] This compound was included because of the observation by workers at the Midwest Research Institute that it seemed to cause damage to Sarcoma 180 in mice used in tumor necrosis screening tests, although it did not cause conspicuous inhibition of the growth of tumors. Goodson, Lewis H., personal communication.

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* Under contract with the U. S. Atomic Energy Commission.

recovered from feces by hot 95% ethanol in a Soxhlet extractor and measured by Beckman Type B spectrophotometer. The absorption peak at 540 $m\mu$ was used for quinolinium compounds and the 469 $m\mu$ peak was used for 2M2PM. Absorption due to normal fecal pigment was taken into account. To measure the dye content of a tissue, such as liver, the sample was washed quickly with alcohol, then minced under a small amount of alcohol. If the pink color of dye appeared in the alcohol, the dye was extracted with the aid of a Soxhlet extractor. A sensitive test for traces of dye in urine and other aqueous solutions was carried out by cutting a thin wick from a disc of filter paper(5) and allowing the solution to rise through this wick and spread through the entire filter paper. Any 4M2MCl, or 2E2E would color the wick. Free dye was removed from urine by passing it through a tube packed with absorbent cotton or by stirring the sample with filter paper. After the dyed cellulose was washed with water and dried, the dye was eluted with boiling alcohol and the quantity measured colorimetrically. In some instances the decolorized urine gradually turned purple on standing, and could be recovered and measured as described. The appearance of pink or purple color could be hastened by heating the sample in a boiling water bath for about 20 minutes, provided the sample was slightly basic. Before heating, therefore, a base was added to urine samples that were acid. Appearance of pink color when a basic decolorized solution was heated, is referred to in this paper as a "heat-pink" test.

Results. *Fecal excretion.* From 36 to 47% of the 4M2M fed, was recovered from feces of Wistar rats, hooded rats, black mice, and brown mice. Practically the same results were obtained whether this dye was administered at concentration of 0.3% or 0.1% in Rockland Mouse Ration or concentration of 0.3% in starch, but a higher excretion was noted when food and dye were ground only 45 minutes. Recovery of 4M2MCl from the feces was about 58%, whereas recovery of 2E2E and 2M2PM was 13 to 27%. No 2M2NPM was recovered, perhaps because of hydrolysis in the stomach. The anil pyridines are usually

readily hydrolyzed by acids, in contrast to the styryl compounds.

Disappearance of dye. The color of dye sometimes persisted 2 to 5 days at sites of subcutaneous injections of 4M2MCl solution in rats. This is in contrast with permanent staining reported to result from subcutaneous injections of 2-(p-acetaminostyryl)-6-(p-acetamidobenzamido) quinoline methoacetate (Styryl 430), reported to produce sarcomata (6). When rats were sacrificed after several days of eating food containing styryl dyes, their tissues did not appear to be stained. However, when a mouse was killed 20 minutes after receiving the last of three 1 ml oral doses of a solution containing approximately 3 mg of 4M2MCl/ml of 5% glucose at 20-minute intervals, 0.8% of the dye was recovered by extraction from the liver, and small quantity was extracted from kidneys. Similar results were obtained at the end of 3 hours, but no dye was found in kidneys and liver of 2 mice and a rat killed 1 day after receiving the solution by stomach tube. The color of saturated solution of 4M2M in 0.9% NaCl became lighter as it passed down the digestive tract of a rat or mouse, until it was yellow in the small intestine. This yellow solution brought about bleaching of dilute solutions of 4M2M or 2E2E within 4 hours. Liquid from the stomach did not produce this effect, nor did liquid from stomach, gall bladder, or intestine of a dog.

Urinary excretion. Rarely a faint trace of dye was observed in urine of animals fed dyed food, but stronger traces were noted following subcutaneous injections. When a rat ate 24.3 mg of 4M2MCl in his food during 2 days, only 0.033 mg of the free dye was found in urine of the first 24 hours and 0.023 mg in the next 24 hours. The additional dye recovered after decolorized urine had stood, was 0.05 mg for each day. After subcutaneous injection of 0.6 mg of 4M2MCl in 5% glucose, 5% of the dye was found in urine. The substance responsible for the color observed after the decolorized dye had stood for some time, had

§ This experiment was planned and executed with the assistance of Dr. Robert A. Woodbury, University of Tennessee, Memphis.

very nearly the same absorption maximum in alcohol as that of a known sample of 4M2M. A negative heat-pink test was obtained with urine of rats, rabbits, hamsters, and a dog that received subcutaneous injections of 4M2MCl solution, but the test was positive after eating of food containing 4M2MCl. The inference that the substance responsible for the heat-pink test was formed in the digestive tract was supported by further observations: A faint pink color was produced when the heat-pink test was applied to samples of 4M2M solution in 0.9% NaCl that had been decolorized in the digestive tract of a rat. A sample of 4M2M solution in 0.9% NaCl, kept over night at room temperature, after addition of a few drops of the liquid from the lower small intestine, then decolorized by passing through cotton, gave a strong heat-pink test. The nature of the substance responsible for the heat-pink test is being investigated further.

Summary. 1. A substantial portion, usually less than half, of the 4M2M, 4M2MCl, 2E2E, and 2M2PM administered to rats and mice in the diet, was found by colorimetric measurements, in the feces. 2. Only a trace of dye color was detected in urine of rats that had received 4M2M or 4M2MCl orally or subcutaneously. 3. Heating, or prolonged

exposure to air, of samples of urine from animals that received 4M2M, 4M2MCl or 2E2E orally, not subcutaneously, produced a pink color. The trace of material responsible for this color seemed to be formed from the dye in the digestive tract. 4. Very little unchanged dye was found in the tissues of rats and mice that had received 4M2M or 4M2MCl orally. 5. A large part of the 4M2M or 4M2MCl administered orally is evidently converted into other substances.

The assistance of Edgar Cress and John Rafter in carrying out the animal experiments is acknowledged with appreciation.

1. Bahner, C. T., Pace, E. S., and Prevost, R., *J. Am. Chem. Soc.*, 1951, v73, 3407.
2. Hughes, B., Bates, A. L., Bahner, C. T., and Lewis, M. R., *Proc. Soc. Exp. Biol. and Med.*, 1955, v88, 230.
3. Lewis, M. R., Hughes, B., Bates, A. L., and Bahner, C. T., *Growth*, 1955, v19, 1.
4. Bahner, C. T., accepted for publication in *Cancer Research*.
5. Saifer, A., Oreskes, I., *Anal. Chem.*, 1953, v25, 1539.
6. Browning, C. H., Gulbrandsen, R., and Niven, J. S. F., *J. Path. Bact.*, 1936, v42, 155.
7. Sheehan, H. L., *ibid.*, 1932, v35, 589.

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Tissue Abnormalities in Newborn Rats from Vitamin B₁₂ Deficient Mothers. (21962)

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Reproductive failure in female rats deficient in vit. B₁₂ has been reported by Dryden, Hartman and Cary(1), Lepkovsky *et al.* (2), Richardson, Witten and Couch(3). Thirty to 60% of the offspring died within 3 days and those which survived were underweight and unhealthy. Ferguson and Couch (4) observed degeneration in heart, liver, thyroid and kidney in chick embryos from hens deficient in B₁₂. This suggested that similar

changes might be found in rat embryos and might account for their early mortality.

Methods. Weanling female rats were placed on B₁₂ low diet of O'Dell *et al.*(5) until 4 weeks before mating when they were transferred to experimental diets. Basal diet was synthetic and contained 25% commercial soybean protein* extracted 6 times by boiling

* The Drackett soybean protein C-1, the Drackett Co., Cincinnati.

Tissues from newborn rats born to vit. B₁₂ deficient mothers compared to those derived from control litters.

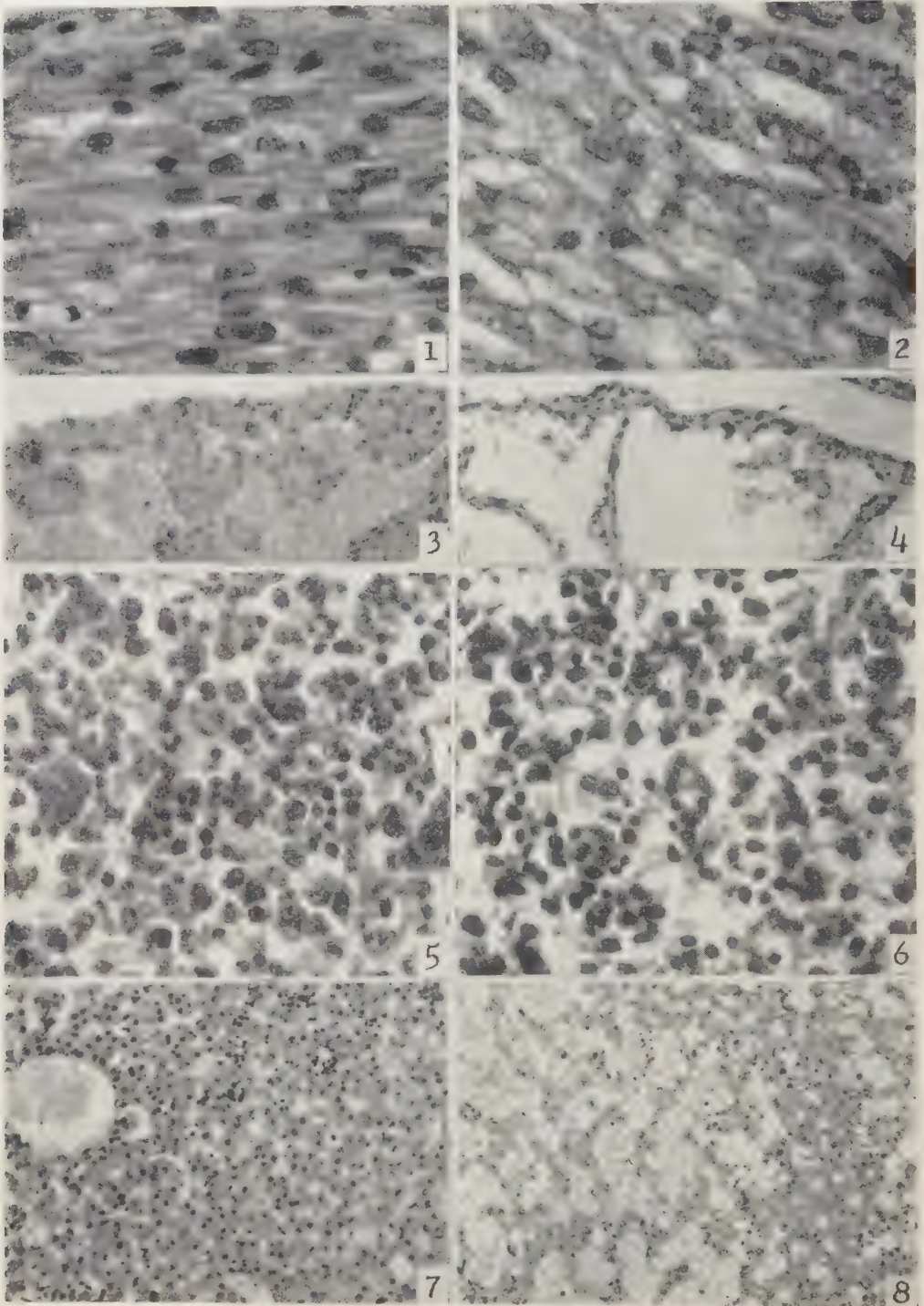


FIG. 1. Longitudinal section of heart muscle from control rat weighing 7.4 g. Chromatic nuclei and basophil cytoplasm. $\times 720$.

FIG. 2. Longitudinal section of heart muscle from experimental rat weighing 2.7 g. Less chromatic nuclei, less differentiated cytoplasm and cell arrangement. $\times 720$.

FIG. 3. Thick wall of auricular appendage from control rat. $\times 450$.

FIG. 4. Very thin auricular wall from experimental rat of 2.7 g. $\times 450$.

FIG. 5. Spleen of control rat with reticular cell background. $\times 720$.

FIG. 6. Spleen of experimental rat (wt 4.15 g) lacking reticular primordial cells and filled with dense pyknotic nuclei with little cytoplasm. Population density of cells is low. $\times 720$.

FIG. 7. Control liver with narrow uniform sinusoids basophil liver cells and numerous blood islands. $\times 450$.

FIG. 8. Liver from experimental rat of 4.7 g showing dilated sinusoids filled with ghost red cells and fat. Note lack of basophilia and diminished size of liver cells. $\times 450$.

70% isopropyl alcohol. One group received basal diet alone. The control group received a supplement of 25 μ g of vit. B₁₂/kg basal diet. The vitamin-deficient females weaned only 40-70% of their young while controls weaned 95%. The young within 3 to 4 hours of birth were killed by ether and injected and fixed whole in either Bouin's solution or neutral formalin, or a mixture of 1 part formalin to 9 parts alcohol for study of glycogen. Ninety young from deficient mothers and 22 from control mothers were used. Hearts, livers, kidneys and spleens were excised, weighed and studied histologically in 7 μ paraffin sections. Other portions were sectioned at 13 μ in freezing microtome. Qualitative histochemical staining tests were used as follows: lipoids; Sudan IV on frozen sections; amyloid; dahlia or methyl violet(6); glycogen; Bauer-Feulgen; ribonucleic acid; toluidin blue with enzyme treated controls; desoxy-ribonucleic acid; methyl green pyronin(7).

Results. Structure and histology of organs of B₁₂ deficient young. Experimental newborn rats were underweight, average 4.75 g (2.14-5.18) while control rats averaged 6.0 g (4.3-8.0). The gestation period for both was 21 days. Weight of heart, liver and kidney of experimental rats showed that whereas the former were below normal weight, they were heavier than normal in proportion to the rest of the body. The above are characteristic of immature animals.

Heart. Aortic arches showed no anomalies, although the chambers of the heart were not examined. The structure of muscle cells in both auricle and ventricle showed a fetal pattern, with little basophil cytoplasm, poorly developed myofibrillae and striations, and marked cell borders (Fig. 1, 2). Sudan staining showed small lipid vacuoles in the cytoplasm. A few lymphocytes were seen in con-

nective tissues. The figures compare longitudinal sections of ventricular muscle photographed under identical conditions. Nuclei were larger but less chromatic with a perinuclear ring of chromatin. D.N.A. determinations showed less nucleoprotein. Auricles were thin, not because of distension but because of failure to develop full thickness of wall (Fig. 3, 4).

Spleen. The control spleen showed a background of reticular cells against which small lymphocytes were contrasted. In the deficient animals the reticular cells had generally disappeared leaving a reticular net of fibers among which were many large, very dense nuclei, often pyknotic. These cells had little cytoplasm. Some cells with more cytoplasm contained hemosiderin (Fig. 5, 6).

Liver. In contrast to the fairly uniform sinusoidal pattern of normal liver, the liver of deficient young under low power showed a dilation of sinusoids between periphery and central veins. The dilated peripheral portions of the sinusoids contained normal non-nucleated red cells. Then followed a zone of red cell ghosts and fragments. The more central portions were plugged with fat globules of undetermined origin. Finally, the zone immediately around the central vein was packed with various leucocytic cells (Fig. 7, 8). This blockage of liver circulation resulted also in shrinkage of liver cords, disappearance of glycogen and nuclei with reduced D.N.A. Von Kupffer cells were all enlarged and contained much blood pigment. Hemopoietic islands of the liver were still present, but the cells, like those of the spleen, had large, dense or even pyknotic nuclei with little cytoplasm. (Fig. 9, 10, 11).

Kidney. The kidney of experimental animals showed retardation of growth more than any other organ. The cortex was of fetal

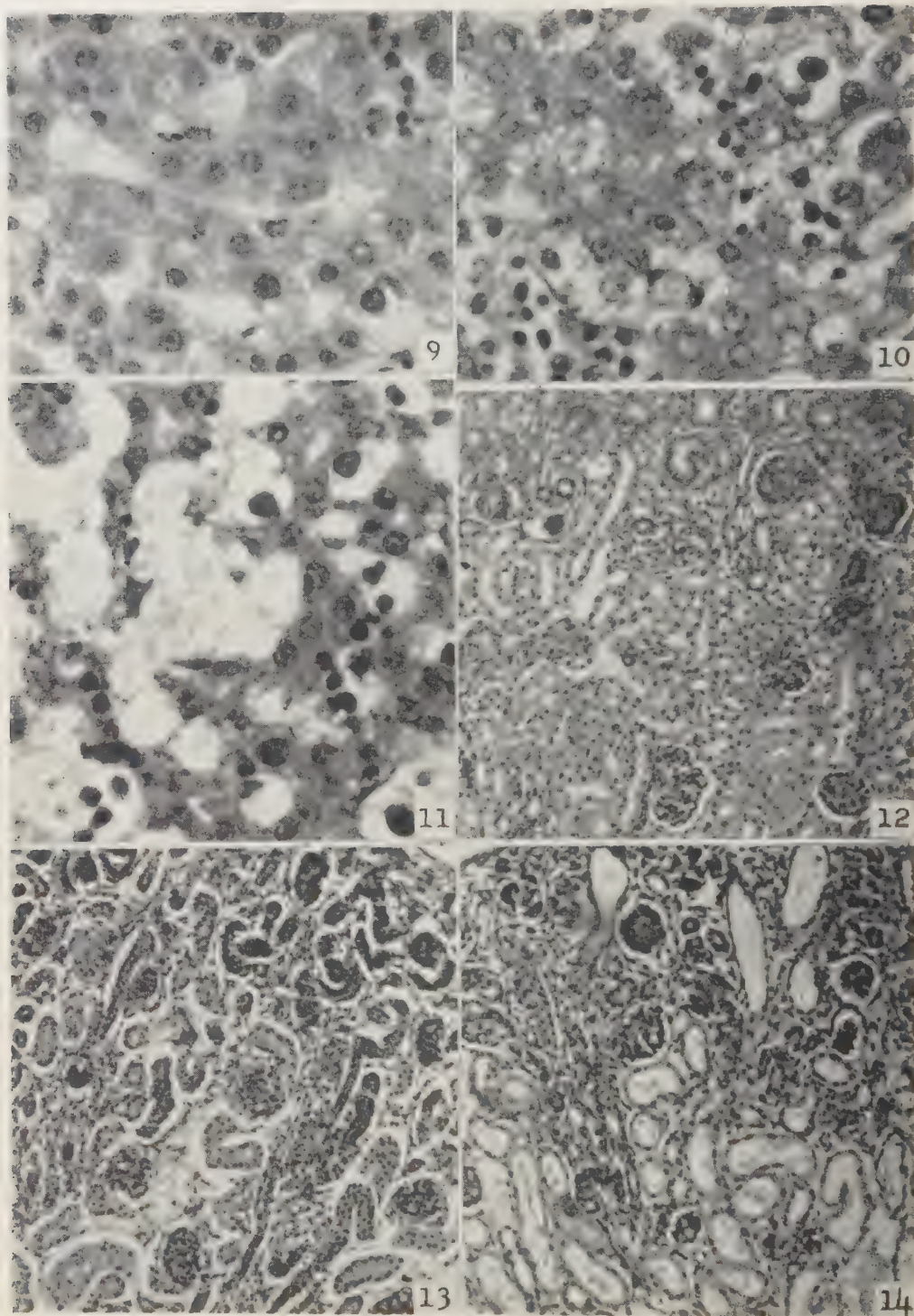


FIG. 9. Control specimen of liver with variable but chromatic nuclei and abundant cytoplasm. $\times 720$.

FIG. 10. Liver from experimental animal of 2.7 g with loss of chromaticity in liver cell nu-

clei, loss of cytoplasmic volume and basophilia and transformation of both blood islands and cells of von Kupffer into pyknotic cells. $\times 720$.

FIG. 11. Liver from deficient rat of 4.2 g wt with great dilation of sinusoids filled with fat and fragmented red cells and fading out of many liver cell nuclei. $\times 720$.

FIG. 12. Cortex from kidney of a control rat (wt 7.4 g) showing one row of undifferentiated glomeruli and tubules. $\times 450$.

FIG. 13. Kidney of deficient rat of 2.7 g showing several laminae of undifferentiated glomeruli and tubules and approximately 2 functional rows deeply placed. Lumens are not clearly defined. $\times 450$.

FIG. 14. Kidney cortex from a deficient rat of 4.05 g showing great dilation of differentiated tubules and reduction of brush border or failure to develop such a border. $\times 450$.

structure filled with incompletely formed glomeruli and tubules. The smallest animals showed the most immature picture (Fig. 12, 13, 14). In addition to the fetal picture, the convoluted tubules of young rats weighing about 4 g showed great dilation of tubules (Fig. 14), the capillary ducts and pelvis of the kidney. The brush border had either sloughed or had never formed. The lumina contained no detritus. Bowman's capsules also were dilated but were still functional. The tubules all resembled distal convoluted tubules. Sudan staining showed fine basal lipid vacuoles in the tubular cells not seen in controls. On the other hand glycogen was lacking. Vascular extravasation was present together with extratubular edema. Newborn young weighing about 2.7 g showed a much less developed kidney in which no lumen was present in the tubules except in deeper, more mature and functioning units (Fig. 13). Here also was much extratubular fluid. In all of these abnormal kidneys there was abundant medullary mesenchyme as in the fetus but no amyloid deposits.

Discussion. It is a general fact that female rats placed on a low B₁₂ diet show little evidence of abnormality in their tissues(8,9) but do develop deficiencies in their reproductive processes and severe growth deficiency in their young(1,3). The degree of growth deficiency and of pathology were coordinate with the immature birthweights, the smallest showing most severe effects. The work of many authors dealing with vit. A deficiency(10,11) with blocking agents like urethane, galactoflavin(12), methylene blue, irradiation(13), etc., show that whatever specificity these agents seem to have lies in the particular moment of susceptibility of tissues at the time of impact of the reagent or deprivation of some essential nutrient.

The case of B₁₂ deficiency in addition to a possible correlation above showed that some of the results seen after birth were the indirect result of blocks in liver or kidneys. Similarly, cardiac anomalies were held responsible for urogenital hypoplasias in the work of Baird *et al.*(12) using galactoflavin.

There may be additional more specific effects in the present experiments because of the probable B₁₂ requirement for synthesis of nucleoprotein(8,9). Hematological work on bone marrow and spleen is needed. Our sections showed great variability in cytoplasmic basophilia in erythrocytes. There was no anemia or general hypoxia in the mothers to indirectly affect the young.

Liver damage has been reported in swine (14) and in adult rat(9) as a result of feeding a B₁₂ deficient diet. Ferguson and Couch(4) reported an increase of fat content in cells of heart muscle, in kidney tubules and in hepatic cells of chick embryos from hens on a diet low in vit. B₁₂. In addition, they reported damage to liver cells beyond this. These cell changes could be prevented by injection of B₁₂ into the egg or into the deficient hens some time before the eggs were laid. The above seem to support the view of Ling and Chow(15,16) that this vitamin directly or indirectly plays a part in the accumulation and transformation of glycogen and fat.

The probable role of vit. B₁₂ in protein metabolism and particularly in nucleoproteins was best seen in heart muscle. The cytoplasmic deficiency could have been due to overwork but was probably a part of the picture of immaturity. The failure to find any amyloid in connective tissues of the body also points to immaturity.

The abnormal changes in the heart, liver, kidney and spleen noted in our experiments with rats were entirely prevented by supple-

menting the deficient maternal diet with vit. B₁₂.

Conclusions. Female rats deprived of B₁₂, from before the time of mating until the end of gestation, produced deficient and weak progeny. This was shown in weights well below normal and arrested development proportionate to the subnormal weights. The defects involved vascular blocks especially in the liver with consequent passive congestion. The heart and kidneys were immature. Proximal convoluted tubules became blocked and transformed into distended tubules histologically like distal tubules. Spleens showed a loss of primitive reticular cells and transformation into large densely chromatic and pyknotic cells. The origin of the fat by which the liver sinusoids were blocked is not known.

1. Dryden, L. P., Hartman, A. M., and Cary, C. A., *J. Nutr.* 1951, v45, 337.
2. Lepkovsky, S. H., Boyson, H. J., Bouthilet, R., Penchary, R., Singman, D., Dimick, M. K., and Robbins, R., *Am. J. Physiol.*, 1951, v165, 79.
3. Richardson, L. R., Witten, P. W., and Couch, J. R., *PROC. SOC. EXP. BIOL. AND MED.*, 1951, v76,

265.

4. Ferguson, T. M., and Couch, J. R., *J. Nutr.*, 1954, v56, 361.
5. O'Dell, B. L., Whiteley, J. R., and Hogan, A. G., *PROC. SOC. EXP. BIOL. AND MED.*, 1951, v76, 349.
6. Pearse, A. G. E., *Histochemistry*, Little, Brown & Co., Boston, 1953.
7. Kurnick, N. B., *Stain Tech.*, 1952, v27, 233.
8. Stern, J. R., Taylor, M. W., and Russell, W. C., *PROC. SOC. EXP. BIOL. AND MED.*, 1949, v70, 551.
9. Seigel, G. B., and Worley, L. G., *Anat. Rec.*, 1951, v111, 597.
10. Wilson, J. G., and Workany, J., *Am. J. Anat.*, 1948, v83, 357.
11. ———, *ibid.*, 1949, v85, 113.
12. Baird, C. D. E., Nelson, M. M., Monic, D. W., Wright, H. V., and Evans, H. M., *Fed. Proc.*, 1955, v14, 428.
13. Rugh, R., *Tr. N. Y. Acad. Sci.*, 1949, v12, 55.
14. Cartwright, C. E., Tatting, B., Robinson, J., Fellows, N. M., Gunn, F. D., and Wintrobe, M. N., *Blood, J. Hematol.*, 1951, v6, 867.
15. Ling, C. T., and Chow, B. F., *J. Biol. Chem.*, 1952, v198, 439.
16. ———, *ibid.*, 1954, v206, 797.

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Accumulation of Homologous Radioiodinated Albumin in Experimental Tumors. (21963)

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Heterologous proteins and amino acids have been shown to localize selectively in transplantable and spontaneous tumors of animals(1,2). This study was undertaken to determine whether homologous proteins are concentrated in tumor tissue since homologous albumin (human serum albumin) tagged with radioactive iodine is used extensively as a diagnostic tracer agent for the detection of brain tumors and metastatic lesions of the liver(3).

Several attempts to measure the distribution of radioactivity in biopsy specimens from tumors in humans have been inconclusive because of low activity in the tissues when per-

missible tracer doses were used. Accordingly, it was decided to study the problem in animals inoculated with experimental tumors, using homologous albumin.

Method. Rabbit albumin was obtained by ammonium sulfate separation, dialyzed and immediately lyophilized. Electrophoretic patterns indicated the material to be albumin, the single characteristic peak being visible. The albumin was trace iodinated with I¹³¹ according to the procedure of Gleason and Tabern (4), and yielded a protein of high specific activity (100-600 μ c/ml with an albumin content of approximately 10 mg/ml). Ultracentrifugation and electrophoretic analysis have

TABLE I. Ratio of Radioactivity of Tumor and Invasive Zone to Normal Liver.

Rabbit No.				Counts/sec./g—			Invasive zone	Tumor
			Day sacrificed	Normal liver	Invasive zone	Tumor	Normal liver	Normal liver
4	No Lugol's	Unperfused	1	5884	8461	12373	1.44	2.10
12	Lugol's	"	1	2938	4891	3661	1.66	1.25
6	No Lugol's	"	2	3191	4589	5535	1.44	1.73
26	Lugol's	"	3	3347	3174	9436	.94	2.82
16	"	"	5	474	618	720	1.30	1.52
						Avg	1.36	1.88
34	Lugol's	Perfused	1	711	894	1398	1.26	1.97
35	"	"	2	337	512	1816	1.52	5.39
30	"	"	3	680	748	2524	1.10	3.72
31	"	"	4	453	494	1745	1.09	3.85
						Avg	1.24	3.73

shown the end product to be similar to native albumin. The tagged albumin was injected intravenously into 9 albino and Dutch rabbits with actively growing Brown-Pearce tumors of the liver. The suspension of Brown-Pearce tumor was obtained by straining a thick homogenate through 2 layers of surgical gauze. Under ether anesthesia a laparotomy was done and about 0.2 ml of the tumor cell suspension was injected into the liver under direct vision. The rabbits were examined daily and after the tumor transplants had become palpable (5-23 days following inoculation), the iodinated albumin was injected intravenously in volumes of 1-5 ml. Some of the animals were given Lugol's solution in their drinking water in order to block their thyroid glands and prevent uptake of inorganic I^{131} from the injected albumin. The animals were divided into 2 groups: the animals of the first group were sacrificed without perfusion 1 to 5 days following the injection of the labelled albumin. They were killed by ether inhalation and autopsied immediately. The other animals were perfused 1 to 5 days following the injection of labelled albumin as follows: under ether anesthesia heparin was injected intraveously and a cannula was inserted into the right external jugular vein. Normal saline was injected into this cannula. Blood was drained through a cannula in the left common carotid artery until only saline was obtained. As soon as that happened, the thorax was opened and normal saline was injected into the descending aorta and drained through the inferior vena cava so as to perfuse the abdominal viscera. Tissue

samples were then removed. Tumor tissue was carefully separated from normal liver tissue and sections through the "invasive" zone were taken. This "invasive" zone consisted of an area at the periphery of the tumor with equal amounts of tumor and normal liver tissue to each side of the center of the section. This was checked by histologic examination. The tissue samples were minced, blotted on filter paper and weighed into culture tubes. They were then dissolved in 2 ml 2N NaOH in a boiling water bath and, after they had cooled, were counted in a sodium iodide (thallium activated) scintillation well counter. The counts recorded were corrected for physical decay to the time of the administration of the dose.

Results. Table I shows the radioactivity expressed as counts/second/gram for normal liver, tumor and "invasive" zone tissue in Brown-Pearce inoculated animals. It can be seen that consistently higher activity is present in tumor tissue than in normal liver tissue. In the perfused animals, this difference appears to be somewhat greater.

Expressed as tumor/normal liver the ratios are 1.25 and 2.82 (average 1.88) in the unperfused animals and 1.97 to 5.39 (average 3.73) in the perfused animals. The ratio of "invasive" zone/normal liver in the unperfused animals ranged from 0.94 to 1.66 (average 1.36); in the perfused animals, it was 1.09 to 1.52 (average 1.24).

Scintigrams(5) showed concentration of radioactive iodine in the liver. There was not enough activity in the tumor, however, to ob-

tain satisfactory differentiation between normal liver and tumor with this method. This is evident from the ratios of tumor tissue/normal liver tissue.

The administration of Lugol's solution to some of the animals did not change the concentration of the iodinated albumin or the ratios between tumor, "invasive" zone and normal liver tissue (Table I).

Comment. Duran-Reynals(1) showed that heterologous proteins and dyes injected intravenously localize selectively in transplantable and spontaneous tumors of mice. He interpreted his findings as, "indicating that newly formed capillaries of tumors are, in general, more permeable than the capillaries of any normal tissue." In our animals, increased concentrations of homologous albumin were demonstrated in tumor tissue as compared to the surrounding normal liver. The ratio of specific activity of tumor/normal liver was even greater after the animals had been perfused. The reason for this may, indeed, be increased permeability of newly formed capillaries with escape of homologous albumin into tumor tissue. Once the albumin has escaped into tumor tissue, it cannot be removed by perfusion. Another possibility is actual metabolic utilization of homologous albumin by the more rapidly growing tumor tissue.

The labelled protein used in our study was

homologous and presumably not denatured. It is assumed that it is handled by the rabbit like native albumin. Whether the intact protein molecule or a metabolite of this protein is deposited in tumor tissue cannot be stated at this time.

Conclusion. The data presented indicate that experimental tumors of the liver (Brown-Pearce) in rabbits accumulate more radioiodinated rabbit albumin than normal liver tissue of the same animal. This increased concentration appears to be unrelated to vascularity since it was demonstrated in perfused animals.

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1. Duran-Reynals, F., *Am. J. Cancer*, 1939, v35, 98.
2. Babson, A. L., and Winnick, Th., *Cancer Res.*, 1954, v14, 606.
3. Yuhl, E. T., Stirrett, L. A., and Libby, R. L., *Ann. Surg.*, 1953, v137, 184; Yuhl, E. T. and Stirrett, L. A., *ibid.*, 1953, v138, 857.
4. Gleason, G. I., and Tabern, D. L., personal communication.
5. Bauer, F. K., Goodwin, Wm. E., Libby, R. L., and Cassen, B., *J. Lab. and Clin. Med.*, 1952, v39, 153.

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Fecal Androgen, A Progesterone Metabolite in the Bovine.* (21964)

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Early investigations clearly indicated that pregnanediol is a product of metabolism of progesterone in man(1-4). Further *in vivo* studies have indicated that progesterone may be reduced to, and excreted as, allopregnanediol-3(α), 20(α)(5) and pregnanol-3(α)-one-20(6). However these metabolites in the urine account for only 5-20% of administered progesterone(7). Recent studies indicate that

pregnanediol is not present in urine of pregnant goats(8) and cattle(9). The importance of a biliary pathway for elimination of progesterone metabolites has been indicated: (a), recovery of pregnanediol from bile of humans after progesterone was given parenterally (10); (b), identification of allopregnanediol-3(β), 20(β) in cattle bile(11); and (c), isolation of pregnanol-3(α)-one-20 and pregnanediol-3(α), 20 (β) from bile of pregnant cows (12). It has been shown in studies of proges-

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terone-21-C¹⁴ metabolism in rats that most of the injected progesterone or its metabolites are carried to the intestine by bile and eliminated in the feces(13). In a study of steroid metabolism in the bovine, we previously determined the neutral ketonic non-alcoholic character of androgens in cow feces(14). Biliary androgenic activity has also been detected in this species(15). However, feces from intact or spayed heifer, following treatment with testosterone, contained comb-growth depressing substances(16). Studies in pregnant cattle have demonstrated a pre-parturient increase in fecal excretion of androgens(17).

These reports led us to suspect that fecal androgen might be related to progesterone metabolism. This paper describes experiments which indicate that fecal androgen in the bovine is dependent on exogenous and/or endogenous progesterone metabolism.

Methods. Progesterone was administered to a 6-months-old Holstein calf, a yearling Jersey heifer, a Jersey cow in 7th month of pregnancy, 3 non-pregnant Jersey cows, and an 18-months-old Holstein bull. Feces were collected before, during, and after progesterone administration; dried in oven at 45°C, and pulverized in a ball-mill. The dried feces from each collection period was then well mixed and 1250 g portions taken for assay. Total weight of feces excreted from day to day did not vary appreciably in a given animal. The androgen content of each fecal sample was determined by measuring comb response of White Plymouth Rock cockerels to the fecal sample incorporated as 10% of control ration. Fecal samples were substituted for an equivalent amount of alfalfa meal present in the control ration(17). Comb response was expressed as 100 times the ratio of comb weight in mg to body weight in g. For comparison, response of chick comb to androstane-3, 17-dione and to Δ 4-androstene-3,17-dione at the level of 10 mg/kg of feed is presented. Recently, androgens present in bovine feces have been identified as androstane-3, 17-dione, Δ 4-androstene-3, 17-dione, and Δ 1,4-androstadiene-3, 17-dione(18). One g of progesterone in olive oil was given subcutaneously, daily, for 3 successive days to each

animal with exception of the pregnant cow which received one g progesterone daily for 5 successive days. Control samples of feces were collected for a 2-day period preceding injections. Fecal samples were then collected during and after progesterone administration on the following days:

	Days
Holstein calf	0-1, 2-4, 5-7, 8-10
Yearling heifer	0-2, 3-5, 6-8
Jersey cow # 544	1, 2, 3, 4, 5, 6
" " # 310	1, 2, 4, 5, 6, 7, 8
" " #10160	1, 2, 3, 4, 5, 6
Holstein bull	1, 2, 3, 4, 5, 6, 7
Pregnant cow	1, 5

Results. Feces of untreated 6-months-old calf exhibited a slight but insignificant androgenic response when fed to baby chicks; whereas marked, significant androgenic responses were obtained from feces of the yearling heifer, 2 of the non-pregnant Jerseys and the pregnant Jersey cow. These data indicate that androgen elaboration in the cow is dependent upon sexual maturation or pregnancy. Feces from the untreated pregnant cow contained the greatest amount of androgenic activity. The peak of fecal androgen excretion thus synchronizes with the period of maximum progesterone production by the animal. It is interesting to note that an insignificant amount of androgen is excreted in feces of the untreated bull as previously reported(19).

Administration of progesterone to the 6-months-old calf, the yearling heifer, the 3 non-pregnant Jersey cows, and the Holstein bull produced statistically significant increases in androgenic content of feces. Upon progesterone withdrawal, the androgen content of feces gradually diminished to or below the pre-treatment levels.

Progesterone administration to the pregnant cow produced a slight but not statistically significant augmentation of androgenic content of feces. A diminution in fecal androgenic activity on prolonged administration of progesterone was indicated in the fecal sample collected after 5 daily doses of progesterone. This slight initial increase as well as the ensuing decrease in androgenic activity on continued progesterone injection might be due to a depression of endogenous progesterone

TABLE I. Excretion of Fecal Androgen in Normal and Progesterone-Treated Bovine.

Animal	Progesterone treatment	Bovine feces		Assay chicks			
		Collections (days)	No. of cockerels	Avg body wt (g)	Avg comb wt (mg)	Comb ratio \pm S.E.	% diff. after inj.
Holstein calf (6 mo old)	Before inj.	2	17	271.2	200.8	74.1 \pm 9.7	—
	After " *	0- 1	19	313.4	327.7	102.8 \pm 10.1	+ 39
		2- 4	18	299.4	306.9	99.6 \pm 12.0	+ 35
		5- 7	15	292.9	223.0	75.2 \pm 7.8	+ 1
		8-10	14	284.6	156.4	54.6 \pm 4.7	- 26
	Control chicks		17	279.6	158.0	55.9 \pm 6.7	
Jersey heifer (yearling)	Before inj.	2	20	311.9	355.5	114.0 \pm 8.1	—
	After " *	0- 2	20	328.2	539.8	162.8 \pm 11.2	+ 43
		3- 5	20	308.0	444.2	144.2 \pm 11.0	+ 27
		6- 8	18	324.4	384.8	120.0 \pm 12.7	+ 5
Jersey cow (pregnant)	Before "	2	15	268.9	401.6	150.6 \pm 17.9	—
	After " †	0- 1	16	240.3	413.0	168.2 \pm 15.8	+ 12
		4- 5	18	263.7	284.2	107.9 \pm 9.9	- 28
	Control chicks		14	283.9	104.9	36.6 \pm 3.5	
	Androgen-fed chicks‡		19	257.7	169.7	65.5 \pm 5.6	
Jersey cow #310	Before inj.	2	21	276.1	166.6	59 \pm 6.3	—
	After " *	1	24	284.5	130.6	46.3 \pm 3.4	- 22
		2	22	290.6	160.4	55.4 \pm 4.0	- 7
		4	24	296.6	264.5	89.3 \pm 8.4	+ 51
		5	24	284.0	158.9	55.4 \pm 4.6	- 7
		6	24	309.1	153.7	50 \pm 5.4	- 15
		7	25	308.6	141.7	46 \pm 3.1	- 22
		8	22	295.8	130.6	44 \pm 3.6	- 25
	Control chicks		23	298.7	158.2	52 \pm 4.4	
	Androgen-fed chicks§		23	293.7	442.8	150.8 \pm 12.2	
Holstein bull	Before inj.	2	22	327.0	200.9	61.8 \pm 5.1	—
	After " *	1	21	320.9	182.7	58 \pm 5.6	- 6
		2	22	337.0	246.9	74 \pm 5.6	+ 20
		3	23	358.0	271	75.6 \pm 6.0	+ 23
		4	22	349.1	534.4	154 \pm 11.4	+149
		5	21	324.2	267.9	82.6 \pm 9.9	+ 34
		6	24	353.7	199.8	56.6 \pm 4.2	- 8
		7	25	334.2	155.8	47 \pm 4.1	- 24
	Control chicks		20	308.6	162.3	52.8 \pm 5.2	
	Androgen-fed chicks§		23	345.6	524.4	149.1 \pm 13.2	
Jersey cow #10160	Before inj.	2	25	248.7	192.0	76.6 \pm 5.4	—
	After " *	1	19	267.6	235.5	87 \pm 5.0	+ 13
		2	24	261.8	189.2	71.9 \pm 4.4	- 6.5
		3	24	256.4	173.5	67.0 \pm 3.8	- 13
		4	23	274.5	292.4	100 \pm 10.1	+ 30
		5	24	269.9	297.9	109 \pm 7.9	+ 42
		6	23	279.4	309.8	110 \pm 8.5	+ 43
	Control chicks		24	276.2	116.1	41.3 \pm 2.0	
	Androgen-fed chicks§		23	271	403	143 \pm 14.5	
Jersey cow #544	Before inj.	2	21	256.5	250	96.9 \pm 5.9	—
	After " *	1	25	264.5	205	75.8 \pm 6.7	- 22
		2	21	289.5	254.4	88.9 \pm 7.0	- 8
		3	21	269.5	288.4	106.4 \pm 7.9	+ 9
		4	22	263.0	280	105.5 \pm 8.4	+ 9
		5	22	278	358	127.1 \pm 10.8	+ 31
		6	20	273.5	318.8	112.0 \pm 13.4	+ 15
	Control chicks		24	252	148.1	58.4 \pm 4.7	
	Androgen-fed chicks§		21	274	410	151.3 \pm 16.9	

* 1 g progesterone admin. daily for 3 successive days. † 1 g progesterone admin. daily for 5 successive days. ‡ Chicks fed androstane-3,17-dione, 10 mg/kg feed. § Chicks fed Δ^4 -androstene-3,17-dione, 10 mg/kg feed. || Third day fecal sample not collected.

TABLE II. Effect of Oral Administration of Progesterone on Male Chick's Comb.

Cone. progesterone in feed (mg/kg)	No. of cockerels	Avg body wt (g)	Avg comb wt (mg)	Comb ratio \pm S.E.
0	23	298.7	158.2	52 \pm 4.4
80	22	312.3	140.6	44.8 \pm 4.9
160	25	306.8	114.1	37 \pm 2.4

secretion which would normally be at a maximum in late pregnancy (Table I).

Discussion. These data demonstrate a statistically significant increase in excretion of fecal androgen in the male and non-pregnant female bovine following injection of progesterone. It is suggested that the androgen found in feces of sexually active bovine female is a metabolite of progesterone and is significantly increased upon injection of exogenous progesterone.

Since androgen is bioassayed by feeding feces to chicks, it has been suggested that progesterone might be excreted as such in the bovine and then converted to an androgen in the chicks' gut, liver or other organ. However, oral administration of progesterone to chicks at a level of 40 mg/kg of feed did not stimulate comb growth (16). This report was verified by the authors at progesterone levels of 80 and 120 mg/kg of feed (Table II).

In general, two possible modes of biogenetic conversion of progesterone to androgens in the bovine may be outlined: (1) Conversion of progesterone by microorganisms of the bovine gastrointestinal tract (2), the 17-hydroxylation of progesterone (adrenal gland?) followed by oxidative degradation of the C₁₇ side chain. Further research is now underway to elucidate these or other possibilities.

Summary. Sexually mature and pregnant cows excrete in their feces appreciable amounts of androgen, whereas males and immature females do not. To determine whether progesterone might be the precursor of the androgen, 7 male and female bovines were given subcutaneously 1 g progesterone daily for 3 or more days. Following these injections

a statistically significant increase in fecal androgen was observed in 6 animals while a non-significant increase was observed in the pregnant cow. These data are believed to indicate that at least a part of the fecal androgen normally found in the sexually active bovine may be derived from progesterone.

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1. Venning, E. H., and Browne, J. S. L., *Endocrinology*, 1937, v21, 711.
2. Buxton, C. L., and Westphal, U., *Proc. Soc. Exp. Biol. and Med.*, 1938, v41, 284.
3. Hamblen, E. C., Cuyler, W. K., and Hirst, D. V., *Endocrinology*, 1940, v27, 169.
4. Dorfman, R. I., Ross, E., and Shipley, R. A., *ibid.*, 1948, v42, 77.
5. Ungar, F., Dorfman, R. I., Stecher, R. M., and Vignos, P. J., *ibid.*, 1951, v49, 440.
6. Venning, E. H., and Ripstein, *Proc. Canad. Physiol. Soc.*, 1947, 43.
7. Sommerville, I. F., and Marrian, G. F., *Biochem. J.*, 1950, v46, 285.
8. Boscott, R. J., *Ciba Foundation Colloquia on Endocrinology*, v2, 327.
9. Hill, D. L., Peterson, W. E., and Cohn, S. H., *J. Dairy Sci.*, 1954, v37, 355.
10. Rogers, J., and McLellan, F., *J. Clin. Endocrinol.*, 1951, v11, 246.
11. Pearlman, W. H., *J. Biol. Chem.*, 1946, v166, 473.
12. Pearlman, W. H., and Cerceo, E., *ibid.*, 1948, v176, 847.
13. Grady, H. J., Elliott, W. H., Doisy, E. A., Jr., Blocklage, B. C., and Doisy, E. A., *ibid.*, 1952, v195, 755.
14. Miller, W. R., and Turner, C. W., *J. Dairy Sci.*, 1953, v36, 296.
15. ———, 1954, unpublished data.
16. Gassner, F. X., *Recent Progress in Hormone Research*, Academic Press Inc., N. Y., 1952, v7, 165.
17. Turner, C. W., *J. Dairy Sci.*, 1948, v31, 1032.
18. Miller, W. R., Turner, C. W., Fukushima, D. K., and Salamon, I. I., The Identification of C₁₉ Steroids in Bovine Feces, *J. Biol. Chem.*, in press.
19. Turner, C. W., *J. Dairy Sci.*, 1947, v30, 1.

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Lack of Synergistic Relationship Between Thyroid and Salivary Gland Function. (21965)

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Kirkwood *et al.*(1-3) have postulated a direct relationship between activity of thyroid gland and ability of salivary glands to clear iodine. This group also believes that salivary glands play an important role in deiodination of such intermediates as diiodotyrosine (DIT) and in the control of the circulating blood level of thyroid hormone. They have demonstrated a monoiodotyrosine iodinase system in rat salivary glands, and concluded that the reverse of this reaction might actually be taking place in the intact glands. To test this hypothesis, Fawcett and Kirkwood(3) followed the deiodination of DIT in salivariectomized and intact rats, and found that DIT was not deiodinated to any appreciable extent in the salivariectomized animals.

Our preliminary observations(4) support the conclusion reached by Freinkel and Ingbar(5,6) that the iodide clearance function of salivary glands is probably not influenced directly by the thyroid, even though the amount of iodide cleared by these glands appears to correlate with the activity of the thyroid. The following data are offered as additional evidence for this conclusion, and for the belief that the deiodination of such thyroid analogues as diiodotyrosine does not take place in the salivary glands.

Methods. Carrier-free doses of I^{131} labeled diiodotyrosine, triiodothyronine, thyroxine and iodide were administered intravenously 15 $\mu\text{C}/\text{kg}$ body weight to mongrel dogs maintained under sodium pentobarbital anesthesia. Saliva samples were collected under Mecholyl stimulation, and chromatographed for 18 hr on paper, together with 20% trichloroacetic acid filtrates of plasma in both 80% phenol and in 68:2:30 butanol-acetic acid-water mixture. In the rat experiments, 50 μC doses of I^{131} diiodotyrosine (DIT) were injected into jugular veins of rats anesthetized with sodium pentobarbital. Blood samples were withdrawn by cardiac puncture and by tail-vein sampling one hour after the DIT injection. 20% trichloroacetic acid filtrates were prepared from these specimens, and chromatographed for 18 hr with the same solvents used in dog experiments. After chromatographs were dry, each strip was cut into 10 mm horizontal sections, and these were counted in a Nancy Wood Scintillation Well Counter to an accuracy of $\pm 5\%$. Activities for each spot were then totalled and used for subsequent calculations of the % deiodination taking place and of iodide clearance.

Results. Irrespective of which of the 4 substances was administered, only I^{131} iodide was

TABLE I. Constancy of Salivary Clearance of I^{131} Iodide Irrespective of whether Iodide or DIT I^{131} Was Administered, and whether Exogenous Thyroid Hormone Was Fed in the Diet.

Time	Clearance*					
	Iodide injected		DIT injected		Iodide injected	
	Dog A	Dog B	Dog C	Dog D	Dog E before thyroid feeding	Dog E after thyroid feeding
15 min.	—	—	33	40	17	19
2 hr	26	20	34	31	19	26
4 "	27	19	20	40	—	—
8 "	22	19	20	25	14	16
12 "	24	20	24	33	—	—
24 "	27	17	—	—	16	21

* Clearance is expressed as cc of plasma cleared of I^{131} iodide per min. and is given by the expression: Clearance = $\frac{(\text{cpm/cc saliva}) (\text{cc saliva/min.})}{(\text{cpm/cc plasma})}$

TABLE II. Rate of DIT Deiodination Determined from Comparison of I¹³¹/DIT Counts on Plasma Paper Chromatograms.

Time	% I ¹³¹ activity appearing as iodide in plasma
30 min.	30
1 hr	59
2 "	70
4 "	75
6 "	75
8 "	80
12 "	78
24 "	80

found on the saliva chromatograms, indicating that thyroid analogues are not cleared by the salivary glands in detectable amounts. By comparing the I¹³¹ iodide activity found on plasma and saliva chromatograms, clearance into the saliva remained relatively constant over a 24 hr period as shown in Table I. Furthermore, feeding of thyroid substance at 0.5% of diet for 2 months, failed to influence the clearance of I¹³¹ iodide in the dog (Table I). Therefore the amount of iodide cleared by salivary glands appears to be a function of the plasma level of iodide, and increased blood levels of thyroid hormone do not alter this clearance.

Kirkwood *et al.* have implied that salivary glands are the principal site of deiodination. We repeated this experiment in salivariectomized and intact dogs. It was found that salivariectomized animals deiodinate DIT at the same rate, and to the same extent as control animals subjected to mock surgery. This deiodination takes place very rapidly, but 10-20% of the circulating activity 24 hr later is still DIT (Table II).

Slices of some tissues and organs of the dog

TABLE III. Relative Distribution of DIT Deiodinase in Tissues of Dog.

Tissue	% I ¹³¹ activity as iodide	Wt of tissue slice (mg)
Liver	17.6	346
Kidney	6.5	404
Spleen	2.3	320
Submaxillary gland	2.5	294
Parotid "	2.4	356
Sublingual "	2.4	289
Skeletal muscle	2.7*	270

* Approximately 2% of Abbott Laboratory DIT preparation is in the form of iodide as determined by our chromatographic methods.

were then incubated in plasma with carrier-free DIT at 37°C. At the end of 3 hour incubation period, trichloroacetic acid filtrates of the media were chromatographed on paper, and the relative amounts of iodide and DIT I¹³¹ determined. Of all tissues analyzed, only the liver and kidneys possessed significant deiodinase activity (Table III). On a weight basis, liver appeared to be more active than kidney, and considering the relatively greater size of this organ, it might be assumed that the liver is the principal deiodination site. To determine this experimentally, however, the deiodination of DIT was followed in plasmas of nephrectomized dogs. It was found that deiodination takes place just as rapidly and completely in the nephrectomized animal as in the control. Additional evidence for the relatively minor role played by the kidney is given by the fact that appreciable amounts of DIT can be found in the urine of intact animals for several hours after the administration of DIT.

Because the data obtained in our dog studies conflict with those of Kirkwood *et al.* for the rat, we attempted to repeat their rat experiments, using the same experimental technics which they described(3). In the first experiments however, we employed cardiac rather than tail-vein blood samples. We also prepared trichloroacetic acid filtrates of plasma and chromatographed these together with the whole plasma samples on the same paper, using the butanol-acetic acid-water mixture employed by Kirkwood. Deiodination took place as rapidly and extensively in salivariectomized rats as in animals subjected to mock surgery. In subsequent experiments, tail-vein samples were compared with cardiac samples taken from salivariectomized and control animals, and it was observed that essentially all the activity present in the tail-vein samples could be attributed to DIT. Our explanation for this discrepancy is that under sodium pentobarbital anesthesia, the animals become acutely cyanotic and the blood pressure falls markedly. Therefore, tail-vein samples are not representative of the systemic circulation, and any DIT passing into the tail might become trapped and never recycled to the liver for deiodination. Slices and homogenates of

the principal tissues of the rat (excluding the thyroid) were also assayed for deiodinase activity. As in the case of the dog, only the liver and kidneys were found to possess activity.

Summary. It is believed that the salivary glands play no active role in metabolism of such thyroid analogues as DIT in the dog. The amount of iodide cleared into the saliva appears to be strictly a function of the circulating plasma level of iodide ion and is not influenced by exogenous thyroid hormone. This clearance will depend of course upon the rates with which exogenous diiodotyrosine, thyroxine and triiodothyronine become deiodinated. Since neither dog nor rat salivary glands appear to contain a DIT deiodinase system such as that found in liver and kidney

tissue, it is concluded that these glands are not involved in the metabolism of DIT nor in the control of the circulating blood level of this analogue.

1. Fawcett, D. M., and Kirkwood, S., *J. Biol. Chem.*, 1954, v209, 249.
2. Thode, Harry G., Jaimet, Charles H., and Kirkwood, Samuel, *New Eng. J. Med.*, 1954, v251, 129.
3. Fawcett, D. M., and Kirkwood, S., *Science*, 1954, v120, 547.
4. Ruegamer, W. R., *Fed. Proc.*, 1955, v14, 274.
5. Freinkel, N., and Ingbar, S. H., *J. Clin. Invest.*, 1953, v32, 1077.
6. ———, *New Eng. J. Med.*, 1955, v252, 125.

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Incorporation in Adrenal Cortex of C¹⁴ Labeled Fractions of *Klebsiella pneumoniae*.*† (21966)

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Crude polysaccharides from *Klebsiella pneumoniae* type B produce severe joint and mild cardiac valvular lesions in the guinea pig(1). To investigate the pathogenesis of the lesions, C¹⁴ labeled polysaccharides have been prepared and the distribution of the label followed in the animal tissues. The present report deals with (a) the adrenal cortical localization of the polysaccharide fractions; (b) comparison of incorporation of the polysaccharide with the whole organism and certain crude protein fractions in the adrenal; and (c) attempts to establish the mechanism.

Methods and materials. *Klebsiella pneumoniae* type B organisms were grown for 16 hours on peptone-glucose agar plates containing either 1-C¹⁴ sodium acetate or uniformly

labeled d-glucose in concentrations ranging from 2 to 25 μ c per ml of media. Bacteria were gently scraped from the agar. Crude polysaccharides were obtained by acid (A) and alkaline (B) extraction technics of Wong (3). The debris (C) remaining after acid extraction was separated into 2 fractions by cold trichloroacetic acid to a concentration of 5% (w/v); the supernatant (D) was dialyzed against running water and evaporated to dryness over a steam bath. A portion of the trichloroacetic acid precipitate (E) was washed with cold trichloroacetic acid, then extracted 2 times with trichloroacetic acid at 90°C for 10 minutes as for isolating nucleic acid (F)(4). Fractions (E) and (G) were insoluble in water and 0.85% sodium chloride and have not been studied in animals. The maximum absorption of the nucleic acid fraction (F) was at 260 m μ . Except for (D), the fractions were dissolved in 1% concentration in 0.85% sodium chloride, autoclaved and injected in less than 1 ml total volume into the ear veins of male and female guinea pigs

* A portion of the data herein presented was given at the meeting of the Fed. Am. Soc. for Exp. Biol.(2), San Francisco, April 1955.

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TABLE I. Incorporation of C¹⁴ into Certain Fractions of *Klebsiella pneumoniae*.

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graph TD
    A["1-C14 acetate (0.5 mc) in agar media (30.0 ml)"] --> B["Whole organisms  
100 mg dry wt"]
    B --> C["Acid extraction"]
    C --> D["Crude bacterial debris (C)  
67.5 mg  
0.320 μc/mg  
21.60 μc total"]
    C --> E["Crude polysaccharide (A)  
32.5 mg  
0.500 μc/mg  
16.25 μc total"]
    D --> F["Cold TCA extraction"]
    F --> G["Soluble"]
    F --> H["Insoluble (E)  
0.223 μc/mg"]
    G --> I["Dialyzable"]
    G --> J["Non-dialyzable (D)  
36.0 mg  
0.0309 μc/mg  
1.116 μc total"]
    H --> K["Hot TCA extraction"]
    K --> L["Soluble (F)  
(nucleic acid)  
0.167 μc/mg"]
    K --> M["Insoluble (G)  
0.125 μc/mg"]

```

weighing 200-250 g at the beginning of the experiment. Whole bacteria were suspended in 0.85% saline, autoclaved and similarly injected. The lethal toxicity of the whole killed bacteria, and the bacterial debris (C), prevented the injection of quantities greater than 1.0 and 0.3 mg respectively. Urine was collected for the first 48 hours and at various daily intervals thereafter. During the collection of the urine, the guinea pigs were placed in glass containers and fed only cabbage supplement instead of their usual stock diet (Purina rabbit pellets). Animals were weighed weekly and sacrificed at varying intervals up to 2 months. Duplicate samples of organs or tissues were taken; one set was used for histologic examination and radioautography, the other set for grinding with distilled water in a Potter homogenizer, plating and counting in the proportional region with a windowless flow counter. The per cent incorporation in tissues or organs is expressed in two ways: (a) per total organ or tissue; (b) per g of dry tissue per kg body weight, ((activity per g tissue/total dose) \times (body weight) \times 100). The latter affords a unit comparison of the activity in different organs and tissues, is useful for tissues of uncertain total weight such as

lymph nodes, and compensates for the gain in organ weights with growth. Radioautographs were made by sandwich technics on no-screen x-ray emulsions with exposures up to 3 months at 4-6°C. This provides fair histologic detail, but unsatisfactory cytologic detail. For comparison with guinea pigs, 6 mice were injected with acid-extracted polysaccharide (A) by tail vein and sacrificed at 1, 3, 7, 14, 30, and 60 days and examined as above. As controls for the labeling of adrenal tissue with noncolloidal material, 1-C¹⁴ sodium acetate, d-glucose, d-sucrose, and dl-sodium glutamate 1-C¹⁴ were heat-sterilized and given alone and with 2 mg of labeled acid-extracted polysaccharide (A) in comparable isotopic amounts. To determine the chemical association of the incorporated crude polysaccharide in the adrenal cortex, the protein and lipid fractions of adrenal homogenates were separated by trichloroacetic acid to 4% concentration (w/v) and ethyl ether extraction respectively. The radioactivity of the various fractions from these 2 procedures was determined.

As a control for reticuloendothelial activity, colloidal Au¹⁹⁸ was injected into 3 animals sacrificed at 1, 3, and 7 days; tissue activities were determined and radioautographs were

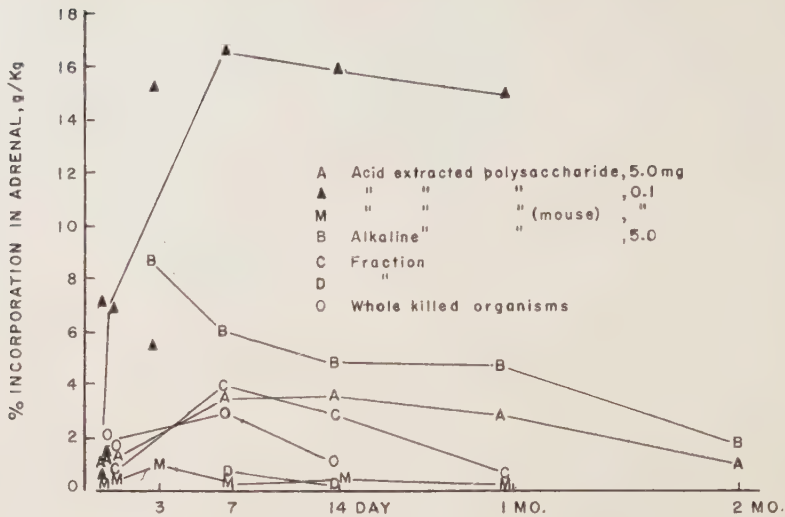


FIG. 1. Comparison of incorporation of various fractions of *Klebsiella pneumoniae* into the adrenal.

made in the same manner as for the other animals. Autoclaved undiluted Higgins india ink in $\frac{1}{2}$ ml quantity was injected into 3 guinea pigs sacrificed at 3, 7, and 14 days; routine microsections were prepared.

Results. Incorporation into fractions of bacteria: The C^{14} of the acetate in the media is incorporated into all of the fractions of the bacteria which have been studied. The crude acid-extracted polysaccharide (A) has the highest activity and the cold TCA-soluble fraction (D), the lowest (Table I). The crude polysaccharide obtained by alkaline-extraction (B) has activity of $0.183 \mu\text{C}/\text{mg}$ in comparison with the crude bacterial debris activity of $0.3955 \mu\text{C}/\text{mg}$. Extraction procedures have not been carried out on this bacterial residue. The polysaccharides obtained by the acid and alkaline extraction techniques have a chemical composition similar to that previously reported (1).

Incorporation into organs and tissues of animals: After intravenous injection in guinea pigs of the various bacterial fractions, little remains in the plasma after 7 days (Table II). There is no appreciable labeling of the erythrocytes or leukocytes. The greatest loss in the urine occurs with the crude bacterial debris (C) and the nucleic acid (F). About the same percentage loss occurs with large as with small doses of the acid-extraction

polysaccharide (A) (Table II). The activity in the urine parallels the activity in the plasma and is not in the form of carbonate or bicarbonate. The organs having the greatest activity, whether on total organ or unit-weight basis, are the adrenal, spleen, liver, lung, and cervical lymph nodes. The latter are consistently higher than the tracheobronchial or mesenteric lymph nodes; the mesenteric node is listed in Table II, for comparison with other studies. After the injection of the 2 crude polysaccharides, (A) and (B), the activity in the adrenal is well maintained for 4 weeks and is present in considerable concentration by the end of 2 months. By contrast, the activity in the spleen, liver, lung, and other organs declines after the first week and is barely detectable at the end of one month and not detectable at 2 months. While the greatest quantity is usually in the liver, the adrenal has by far the highest concentration per gram of tissue. The radioautographs reveal this activity to be in the zona fasciculata of the cortex and to be absent in the medulla (Fig. 2). In animals dying during the first day, the adrenals are engorged and microsections occasionally disclose zones of hemorrhage and minute areas of necrosis, usually in the inner cortex. In animals sacrificed at subsequent intervals, no histologic changes are detectable even though the radioactivity is relatively

TABLE II. % Incorporation in Guinea Pig Tissues at Optimal Time, 1 and 2 Weeks.

I.V. dose, mg	Weeks	Plasma		Adrenal		Spleen		Liver		Lung		Mesenteric lymph node,		Pituitary, g/kg	Loss in urine, %
		Total	g/kg	Total	g/kg	Total	g/kg	Total	g/kg	Total	g/kg	Total	g/kg		
Acid extraction polysaccharide (A)	1	.14	.08	1.0	5.0	2.6	6.6	27.6	2.4	.6	.4	1.7			12
	2	.10	.05	1.2	6.1	1.4	3.5	11.9	1.0	1.1	.8	.3		.3	14
Whole, killed bacteria	1	.09	.05	3.6	17.9	.8	2.0	18.9	1.6	.2	.1	.9		.7	7
	2	.00	.00	4.5	22.2	1.2	3.1	8.0	.7	.0	.0	.0			14
Alkaline extraction polysaccharide (B)	1	.11	.06	.8	3.9	.6	1.7	10.9	.9	.4	.3	.1		2.2	6
	2	.04	.02	.2	1.2	.4	1.1	6.0	.5	.1	.1	.1		.0	8
Bacterial debris (C)	1	.17	.09	1.8	8.6	.4	1.1	2.5	2.1	.5	.3	.1		.0	14
	2	.03	.02	1.4	6.6	.3	.7	2.8	2.4	.2	.2	.2		.0	17
Nucleic acid (F)	1	.25	.13	.8	4.1	.5	1.2	13.6	1.2	.2	.1	.1		.1	26
	2	.16	.09	.5	2.6	.3	.7	8.1	.7	.0	.0	.1		.0	73
TCA soluble (D)	1	.03	.14	.0	.1	.0	.0	.0	.0	2.0	.1	.0		.0	31
	2	.00	.00	.0	.4	.0	.0	.0	.0	.4	.3	.0		.0	27
	1	.07	.05	.1	.7	.4	1.0	4.9	.4	.1	.1	.1		.0	16
	2	.00	.00	.1	.6	.2	.4	3.5	.3	.0	.0	.2		.0	24

high. The simple extraction procedures with water, fat solvents, and trichloroacetic acid show there is no significant labeling of lipid, but do not establish whether the labeled substances are protein or carbohydrate or both. Further investigation is, of course, needed to determine the nature of the labeled moieties. There is no appreciable adrenal incorporation of C¹⁴ from glutamic acid, glucose, sucrose, or acetate. In the spleen, the activity is in the red pulp, but not the Malpighian bodies and in the lymph nodes the radioactivity is present in the reticuloendothelial areas and not the lymph follicles. The distribution in mice is not the same as in guinea pigs, there being a greater activity in a gram of liver than of the adrenal or spleen.

India ink and colloidal gold: Variable sized particles of carbon from the india ink are found in the lung, liver, and spleen in the expected area. Some thromboses of pulmonary arteries and capillaries contain considerable black pigment. Fine black particles occur in the hepatic cord cells. Only traces of pigment can be detected in the microsections of adrenal. This pigment is in the sinusoidal epithelium and rarely in the cortical cells. The total activity and amount of colloidal gold given to the guinea pigs is not recorded, but the ratios of organ activities per gram dry weight at one week are as follows: Adrenal 1.0, spleen 23.36, liver 14.2, lung 0.13, mesenteric node 0.087, and pituitary 0.0157. Thus, colloidal gold does not have the distribution of the bacterial fractions and differs from the distribution of india ink particles in the lung and adrenal.

Discussion. The distribution of C¹⁴ labeled polysaccharides in the guinea pig is similar to that observed in mice and rabbits with I¹³¹ and dye-tagged antigen(5-7) and with fluorescent antibody(8) with the exception of a remarkably high concentration in the adrenal cortex of the guinea pig. In current theory there are three mechanisms for incorporation of the C¹⁴ label into the adrenal cortex: (a) cellular phagocytosis and storage of certain large molecular moieties of the non-homogeneous bacterial fractions, (b) incorporation of a simple molecular constituent into a protein or carbohydrate component of

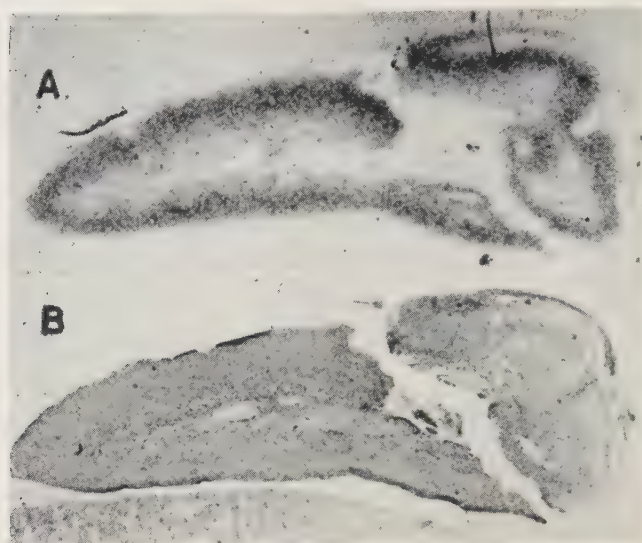


FIG. 2. Radioautograph of adrenal: (A.) of guinea pig given 5.0 mg of acid-extracted polysaccharide. Comparison with H & E stained microsection. (B.) reveals activity to be in outer cortex with slight activity in inner cortex and no activity in medulla. Exposure 6 wk on no-screen x-ray film.

the adrenal cortical cells, and (c) metabolic utilization of some simple molecular constituents such as a hexuronic acid.

The long persistence of the radioactivity in the adrenal argues against a metabolic utilization of a simple molecular constituent. The radioactivity also persists in the adrenal while disappearing from the accepted sites of more active reticuloendothelial activity such as spleen and liver. Studies on the behavior of colloidal substances do not clarify the appearance of the label in the adrenal cortex. Size alone has some bearing upon the localization in the different tissues of the reticuloendothelial system(9,10), particles of larger size showing rapid deposition in the liver and spleen and colloids of smaller particle size being deposited more slowly but in greater concentration in the bone marrow and spleen. In the former study(9), the adrenals contained only 0.002% of injected dose as compared to the bone marrow value of 44.0; spleen, 1.3; and liver 3.7%. Further study of the nature and behavior of colloids is needed to explain the differences in localization and storage of protein and polysaccharide antigens and of the various colloids of greater particle size. The values in mice as well as guinea pigs are quite different from the observations of anti-

gen localization of Friedlander's capsular polysaccharide in mice(8) and of I^{131} protein antigens in rabbits(7).

The present study shows a rough parallelism between the degree of labeling of the bacterial fraction and the concentration of C^{14} in the adrenal. For example, the acid-extraction polysaccharide (A) comprises 32.5% by dry weight and 42.9% of the C^{14} in the bacteria, while the TCA-soluble fraction (B) of debris represents 36% by weight and 0.29% of the radioactivity. Depending on dosage given, the optimal polysaccharide label incorporation (% g/kg) in the adrenal is 6 to 22 while that of fraction (D) is but 0.7 (Table II). As yet a similar parallelism between reducing sugar content of the bacterial fraction and the concentration in the adrenal has not been established, but some chemical affinity of the adrenal cortex, whether for colloids or smaller molecules, is apparent.

The incorporation of such foreign bacterial products may prove to be of considerable significance in so-called stress responses, in the electrolyte disturbance and carbohydrate metabolism with infections, antigen antibody relationships, "collagen-vascular" diseases, and in ascorbic acid metabolism.

Summary. In the guinea pig the adrenal

cortex is the site of the greatest concentration of C^{14} after the intravenous injection of labeled polysaccharide and protein fractions of *Klebsiella pneumoniae*. This concentration persists for two months, while the activity of liver, spleen, lung and other organs declines and disappears. In general, the bacterial fraction with the highest incorporation of C^{14} from the media yields the greatest incorporation in the adrenal.

1. Jones, R. S., Carter, Yolande, and Rankin, J. deW., *Brit. J. Exp. Path.*, 1954, v35, 519.
2. Jones, R. S., and Carter, Yolande, *Fed. Proc.*, 1955, v14, 407.
3. Wong, S. C., *PROC. SOC. EXP. BIOL. AND MED.*, 1938, v38, 107 & 110.

4. Gale, E. F., and Folkes, J. P., *Biochem. J.*, 1953, v53, 483.
5. Dixon, F. J., Bukantz, S. C., Dammin, G. J., and Talmage, D. W., *Fed. Proc.*, 1951, v10, 553.
6. McMaster, P. D., and Kruse, H., *ibid.*, 1951, v10, 564.
7. Latta, H., Gittlin, D., and Janeway, C. A., *Arch. Path.*, 1951, v51, 260.
8. Hill, A. G. S., Deane, H. W., and Coons, A. H., *J. Exp. Med.*, 1950, v92, 35.
9. Dobson, E. L., Gofman, J. W., Jones, H. B., Kelley, L. S., and Walker, L. A., *J. Lab. & Clin. Med.*, 1949, v34, 305.
10. Zilversmit, D. B., Boyd, G. A., and Brucer, M., *ibid.*, 1952, v40, 225.

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Thioctic Acid Content and Pyruvate Oxidation in Ethionine-Damaged Rat Livers.* (21967)

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Recent investigations have established the role of alpha-thioctic acid (lipoic acid) (6,8 dithio-octanoic acid) as essential cofactor for metabolism of alpha-keto acids in both bacteria and animal tissue. This vitamin was first described by Kidder and Dewey as "Protogen," an essential growth factor for *Tetrahymena geleii*, present in liver and yeast; more recently its identity with "Pyruvate Oxidation Factor" of O'Kane and Gunsalus and "Acetate Replacing Factor" of Guirard has been established. The compound has been crystallized from liver and chemically synthesized. Thioctic acid is essential for oxidation of pyruvate by cells of *Streptococcus fecalis*, grown on thioctic acid-free medium, and is intimately associated with pyruvic oxidase and alpha-keto glutaric oxidase recovered from pigeon breast and pig heart muscle respectively. The role of this compound has been

defined in the transfer to coenzyme A of the 2-carbon acetyl fragment obtained by oxidative decarboxylation of pyruvate and the 4-carbon succinyl fragment resulting from oxidative decarboxylation of alpha-keto glutarate. These developments have recently been extensively reviewed by Reed(15) and Gunsalus(7). The role of thioctic acid in animal nutrition has not been defined. No symptoms can be induced, nor can growth be diminished, in rats, by feeding a diet deficient in thioctic acid and simultaneously depressing possible intestinal bacterial synthesis of thioctic acid by feeding antibiotics(21). Feeding of an antimetabolite, the 8-methyl analogue of thioctic acid, which inhibits growth for some bacteria, produces no symptoms in chicks and rats(22). Impaired alpha-keto acid metabolism in severe human hepatic dysfunction (1, 2,19) and in experimental hepatic necrosis in rats(12,16) has been reported, and the importance of sulfur-containing amino acids in maintaining integrity of the liver is well recognized(5,16).

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[†] Fellow of the National Foundation for Infantile Paralysis.

Therefore it was decided to investigate thioctic acid content of liver and its relationship to pyruvate metabolism in hepatic injury produced in rats by prolonged feeding of ethionine(9). This amino acid is the ethyl analogue of methionine and interferes with utilization of methionine in protein synthesis(18).

Method. Female rats of the Wistar strain, weighing 110 to 150 g, were placed on following diet, to which dl-ethionine was added to a final concentration of 0.5%: Sucrose 75%, vitamin-free casein (Nutritional Biochemical Corp.) 16%, salt mixture (HMW) 4%, and corn oil 5%. Thiamine 10 mg, niacinamide 40 mg, pyridoxine 10 mg, riboflavin 20 mg, calcium pantothenate 40 mg, choline chloride 300 mg, menadione 5 mg, Vit. A 60000 units, and irradiated ergosterol 8500 units were added to each kg of diet. Other rats were simultaneously maintained on above diet without ethionine and were either fed *ad libitum* or pair-fed in amounts equal to those consumed by ethionine-fed animals. After 21 to 35 days on the diet, and after a 16- to 20-hour fast, the rats were killed by decapitation. At sacrifice, blood was collected from the neck and pyruvic acid determined by the method of Friedemann and Haugen(4). Livers were immediately excised and homogenized in 9 parts of chilled isotonic sucrose in glass homogenizer at 4°C. The homogenate was centrifuged 10 minutes at 600 x g to sediment nuclei and cellular debris. Supernate was then centrifuged at 9000 x g for 10 minutes to precipitate the mitochondria. The mitochondrial preparation was

resuspended in 2 ml of isotonic sucrose per original gram of wet liver and reprecipitated at 14000 x g for 10 minutes. Mitochondria were then resuspended in isotonic KCl. Both kidneys were also excised and homogenized in 15 ml distilled water. Mitochondrial pyruvate oxidation was measured by the Warburg method, with flask contents as in Table I. Mitochondrial protein was determined, in quadruplicate, by the method of Lowry *et al.* (11). Thioctic acid determination was accomplished, according to the method of Stokstad *et al.*(20), by turbidometric assay of the stimulation of growth of *Corynebacterium bovis*.† The original medium was modified to stimulate maximal growth by addition of 1.5 g of enzyme-hydrolyzed casein/liter of double strength medium. The enzyme hydrolyzed casein (Trypticase-Baltimore Biological Laboratories) and acid hydrolyzed casein (Vitamin-free Casamino Acids-Difco) were adsorbed with charcoal, extracted with benzene at pH 3, and then reabsorbed with charcoal at pH 6 to remove any residual thioctic acid. Dl-methionine, 0.5 g, was added to each liter of double strength medium to counteract any inhibition of growth of test organism which might be produced by ethionine, as previously described for *Escherichia coli*(8). However, no significant inhibition of growth of *Corynebacterium bovis* due to ethionine, at concentrations ranging from 10^{-3} to 10^{-5} M, could be detected. To free biologically-inactive, bound thioctic acid(6,13), 1 ml. aliquots of liver or kidney homogenate and 1 ml aliquots of mitochondrial suspension containing 5 to 10 mg of protein/ml were autoclaved with 1 ml of 12 N H_2SO_4 for 3 hours at 120°C and 15 lbs pressure. The digests were neutralized with concentrated KOH and diluted to final concentration of 0.4 to 4.0 mγ of d-alpha-thioctic acid/ml. Urines, collected from fasting rats kept in metabolism cages for 48 hours, were diluted to a similar concentration. The diluted samples were filtered and 2.5 ml aliquots were added to 2.5 ml of double strength medium, in quadruplicate, so that final concen-

TABLE I. Hepatic Mitochondrial Pyruvate Oxidation.*

Diet	No. of animals	Mean QO_2 /mg protein \pm S.E.
Control, fed <i>ad lib.</i>	3	66 \pm 5.8
" , pair fed	7	69 \pm 2.7
Ethionine	13	36 \pm 1.3

* Results expressed as μ l O_2 taken up/hr/mg mitochondrial protein (based on oxygen uptake in first 10 min.).

Flask contents: Na pyruvate .02 M; Na fumarate 6.7×10^{-4} M; KCl .05 M; $Mg SO_4$.002 M; ATP .001 M; DPN 2×10^{-4} M; Niacinamide .01 M; Cytochrome C 1×10^{-5} M; PO_4 .02 M, pH 7.35. Mitochondrial suspension containing 8-12 mg protein.

Flask vol.: 3 ml, 20% KOH in center well.

Temperature, 37°; gas phase, O_2 ; thermequilibrium, 5 min.

† We are indebted to Dr. E. L. R. Stokstad of the Lederle Division, Amer. Cyanamid Co., for the stock culture of *Corynebacterium bovis* and generous supplies of dl- α -thioctic acid.

TABLE II. Hepatic Mitochondrial Oxidation*

Diet	No. of animals	Substrate		O ₂ uptake due to added pyruvate
		Pyruvate 60 μ M Fumarate 2 μ M	Fumarate 2 μ M	
Control, pair fed	5	69 \pm 2.8	32 \pm 2.4	37 \pm 2.7
Ethionine	6	37 \pm 2.2	28 \pm 3.0	9 \pm 2.7

* Results expressed as mean μ l O₂ uptake/hr/mg mitochondrial protein \pm S.E. Flask contents as per Table I, with pyruvate omitted as indicated.

tration of d-alpha-thioctic acid ranged from 0.2 to 2.0 m γ /ml. The tubes were then inoculated with one drop of a saline-washed 48-hour culture of *C. bovis*, grown in basal medium enriched with thioctic acid. Inoculated tubes were incubated 88 hours at 30°C, and growth measured turbidimetrically in a photospectrometer. Optical density, prior to inoculation, of the relatively dark solutions obtained by liver digestion, was subtracted from the optical density following incubation, to find the increment in turbidity due to bacterial growth. A standard curve, obtained by measuring growth resulting from addition of known amounts of synthetic dl-alpha-thioctic acid[†] (having one-half the biologic activity of naturally occurring d-isomer) to the basal medium, was established simultaneously for each group of samples. At final concentrations of 0.2 to 2.0 m γ d-alpha-thioctic acid/ml, values obtained from unknowns were reproducible without a constant tendency to upward or downward "drift"; values falling outside above range were discarded.

Results. Rats fed the diet containing ethionine lost weight more rapidly than pair-fed controls. At autopsy, the livers were grossly enlarged, pale, firm, and granular. Microscopic examination revealed lobular disarrangement, marked variation in cell size, cellular swelling and cytoplasmic coagulation, with marked inflammatory cell and fibroblastic infiltration and slight fibrosis, as previously described by Koch-Weser and Popper (9). Kidneys were enlarged, soft, and pale. Microscopic examination revealed marked tubular hydropic degeneration. A similar lesion occurring following acute injection of ethionine has been described (23).

Impaired pyruvate metabolism in intact animals, following prolonged ethionine feeding, was suggested by a significant ($p < .02$)

terminal elevation in fasting blood pyruvate levels from $2.9 \pm .39$ mg% in pair-fed controls to $4.2 \pm .29$ mg% in ethionine-fed animals. Pyruvate oxidation by mitochondrial preparations from livers of animals fed ethionine was markedly diminished (Table I). While mitochondria from livers of control animals maintained uniform pyruvate oxidation rate for over 40 minutes, the rate of pyruvate oxidation by mitochondria obtained from livers of ethionine-fed animals declined progressively during each 10 minutes of incubation. Hourly oxygen uptake per mg of mitochondrial protein was calculated on the basis of oxygen consumption during the first 10 minutes, to minimize above difference. The endogenous oxygen consumption by mitochondria in absence of substrate was negligible. When pyruvate was omitted from the flasks, the sole remaining substrate was 2 μ moles of fumarate "sparker" per flask. When oxygen uptake in presence of fumarate alone was subtracted from oxygen uptake in presence of fumarate plus pyruvate, the added oxygen consumption due to presence of pyruvate was markedly greater in mitochondria from livers of control animals than in ethionine-fed rats (Table II). These differences were highly significant ($p < .001$).

The thioctic acid content of liver per gram wet weight was essentially similar for rats fed chow *ad libitum*, and for rats fed ethionine-free diet *ad libitum* and in limited amounts (Table III). The mean value for control animals of 1.2 γ of d-alpha-thioctic acid per g wet liver is of the same order of magnitude as previously reported values (6,13). However, a marked decrease in thioctic acid content to 0.4 γ /g was found in livers of ethionine-fed rats. When the observed thioctic acid content was corrected for the increase in liver size noted in ethionine-fed rats, by expressing the

TABLE III. Thioctic Acid Content* of Rat Organs.

Diet	Liver			Kidney		
	No. of animals	γ /g wet wt	γ /100 g initial body wt	No. of animals	γ /g wet wt	γ /100 g initial body wt
Chow	7	$1.2 \pm .10$	—	—	—	—
Control, fed <i>ad lib.</i>	13	$1.1 \pm .07$	$4.0 \pm .27$	6	$1.1 \pm .12$	$1.2 \pm .11$
" , pair fed	10	$1.2 \pm .12$	$4.6 \pm .38$	9	$1.4 \pm .10$	$1.3 \pm .10$
Ethionine	15	$.4 \pm .05^\dagger$	$1.7 \pm .21$	9	$1.3 \pm .11$	$1.3 \pm .10$

* Results expressed as γ of d- α -thioctic acid/g of wet organ and as γ of total liver or kidney. d- α -thioctic acid/100 g of rat body wt prior to beginning experimental diet. (Mean \pm S.E.)

† For difference between livers of pair fed controls and ethionine-fed animals, $p < .001$.

results in terms of total liver d- α -thioctic acid/100 g original body weight, prior to starting on diet, the presence of an absolute decrease in hepatic thioctic acid from 4.6 to 1.7 γ of d- α -thioctic acid per 100 g of original body weight was established, at a highly significant ($p < .001$) level (Table III).

Thioctic acid content of mitochondria obtained from livers of ethionine-fed animals was also significantly ($p < .001$) diminished, from 27 to 8 m γ of d- α -thioctic acid/mg of mitochondrial protein (Table IV). Hepatic depletion of thioctic acid was apparently uniform in the mitochondria and in other portions of the cell, since the percentage of total hepatic thioctic acid which could be recovered from mitochondria was approximately equal in both control and experimental animals. The values of 67 and 62% respectively coincide closely with the 64% reported by Reed and Cormier(14).

Kidney was found to contain approximately 1.3 γ of d- α -thioctic acid/g wet tissue; this value approximately equals the amount found in liver and confirms the observations of Reed, *et al.*(13) who found approximately equal amounts of "Acetate Replacing Factor" activity in rat liver and kidney. No significant relative or absolute diminution of renal thioctic acid content could be detected in animals fed ethionine (Table III), in spite of anatomic damage to the kidneys. This is compatible with previous reports that the chemical dysfunction induced by ethionine intoxication is not as severe in kidney as in liver, the incorporation of amino acids into proteins following ethionine injection being much more impaired in liver than in kidney(18).

The 24-hour urine excretion of thioctic acid

was slightly greater in fasting ethionine-fed rats than in pair-fed controls. The values of 330 and 230 m γ of d- α -thioctic acid per 24 hours respectively fall within the normal range of 100 to 500 m γ per 24 hours previously observed by Stokstad, *et al.*(21). The difference between the 2 groups, however, is significant only at the .05 level. Apparently, a general impairment of thioctic acid synthesis does not exist in ethionine-fed rats.

Diminished pyruvate oxidation by liver mitochondria from ethionine-fed rats could not be restored by *in vitro* addition of 1 γ of dl- α -thioctic acid to the flask mixture. Injection of 60 γ of dl- α -thioctic acid into ethionine-fed rats at 96, 72, and 48 hours prior to sacrifice did not increase hepatic mitochondrial thioctic acid content above that found in untreated animals (Table IV).

Discussion. The demonstrated defect in mitochondrial pyruvate oxidation is probably not due to thioctic acid depletion. It is known that thiamine pyrophosphate, diphosphopyridine nucleotide, thioctic acid, coenzyme A, and specific protein apooxidases are all required to maintain pyruvate oxidation(15). DPN has been observed to diminish in livers of ethionine-fed rats(17). Coenzyme A originates in part from methionine and cystine (24) and is diminished in livers of rats fed a diet deficient in sulfur-containing amino acids (3, 12). Since ethionine interferes with normal metabolism of methionine, it is possible that the coenzyme A content of mitochondria is also diminished. Moreover, ethionine diminishes protein synthesis(18) and is incorporated in synthesized abnormal proteins (10); therefore the apoenzymes required for pyruvate oxidation may be deficient. In spite of the observed depletion of mitochondrial

TABLE IV. Thioctic Acid Content* of Liver Mitochondria.

Diet	No thioctic acid injection			Inj.† with thioctic acid	
	No. of animals	Mitochondrial thioctic acid content	% of total liver thioctic acid recovered from mitochondria	No. of animals	Mitochondrial thioctic acid content
Control, fed <i>ad lib.</i>	5	32 ± 2.7	—	—	—
" , pair fed	10	27 ± 2.6	67 ± 8	3	25 ± 3.0
Ethionine	10	8 ± 1.0	62 ± 5	3	6 ± 1.5

* Results expressed as mγ d-α-thioctic acid/mg mitochondrial protein (mean ± S.E.).

† Injected with 60 γ dl-α-thioctic acid 96, 72, and 48 hr prior to sacrifice.

thioctic acid in these animals, addition of thioctic acid *in vitro* does not increase the diminished pyruvate oxidation observed in liver mitochondria. Probably, a variety of factors other than thioctic acid depletion probably contribute to the pyruvate oxidation defect.

A general impairment of thioctic acid synthesis does not exist in ethionine-fed rats and therefore cannot account for diminished hepatic thioctic acid content. Since thioctic acid injection does not increase thioctic acid content of liver mitochondria in ethionine-fed rats, it appears possible that thioctic acid binding mechanism of liver may be damaged by ethionine feeding, possibly due to impaired synthesis of apoenzymes to which thioctic acid is bound, or due to impaired synthesis of enzymes which catalyze protein binding of thioctic acid or its conversion to the form in which it is biologically active.

Summary. Terminal blood pyruvic acid level was elevated in rats with liver lesions induced by feeding a diet containing ethionine. Pyruvate oxidation by mitochondria from livers of these rats diminished. The hepatic content of thioctic acid was decreased relative to control animals; the thioctic acid content of liver mitochondria was also reduced. *In vitro* addition of thioctic acid did not stimulate the diminished hepatic mitochondrial pyruvic acid oxidation. Undiminished urinary excretion of thioctic acid, failure of thioctic acid injection to increase the diminished hepatic mitochondrial thioctic acid content, and normal kidney thioctic acid content suggest that total body thioctic acid synthesis is unimpaired but that hepatic thioctic acid "binding" is impaired, in rats fed a diet containing ethionine.

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1. Amatuzio, D. S., and Nesbitt, S., *J. Clin. Invest.*, 1950, v29, 1486.
2. Carfagno, S. C., De Horatius, R. F., Thompson, C. M., and Schwarz, H. P., *New Eng. J. Med.*, 1953, v249, 303.
3. Chernick, S. S., Moe, J. G., and Schwarz, K., *Fed. Proc.*, 1955, v14, 191.
4. Friedemann, T. E., and Haugen, G. E., *J. Biol. Chem.*, 1943, v147, 415.
5. Goettsch, M., *Ann. N. Y. Acad. Sci.*, 1954, v57, 839.
6. Gunsalus, I. C., Struglia, L., and O'Kane, D. J., *J. Biol. Chem.*, 1952, v194, 859.
7. Gunsalus, I. C., *J. Cell. Comp. Physiol.*, 1953, v41, supp. 1, 113.
8. Harris, J. S., and Kohn, H., *J. Pharm. and Exp. Therap.*, 1941, v73, 383.
9. Koch-Weser, D., and Popper, H., *Proc. Soc. Exp. Biol. and Med.*, 1952, v79, 34.
10. Levine, M., and Tarver, H., *J. Biol. Chem.*, 1951, v192, 835.
11. Lowry, O. H., Rosebrough, N. J., Farr, L. A., and Randall, R. J., *ibid.*, 1951, v193, 265.
12. Olson, R. E. and Dinning, J. S., *Ann. N. Y. Acad. Sci.*, 1954, v57, 889.
13. Reed, L. J., DeBusk, B. F., Johnston, P. M., and Getzendaner, M. E., *J. Biol. Chem.*, 1951, v192, 851.
14. Reed, L. J., and Cormier, M. J., *Proc. Soc. Exp. Biol. and Med.*, 1951, v77, 724.
15. Reed, L. J., *Physiol. Rev.*, 1953, v33, 544.
16. Schwarz, K., *Ann. N. Y. Acad. Sci.*, 1954, v57, 878.
17. Shank, R. E., Mendeloff, A. I., and Karl, M., *J. Clin. Invest.*, 1953, v32, 603.
18. Simpson, M. V., Farber, E., and Tarver, H., *J. Biol. Chem.*, 1950, v182, 81.
19. Smith, L. H., Ettinger, R. H., and Seligson, D., *J. Clin. Invest.*, 1953, v32, 273.
20. Stokstad, E. L. R., Hoffman, C. E., and Belt,

M., PROC. SOC. EXP. BIOL. AND MED., 1950, v74, 571.

21. Stokstad, E. L. R., Broquist, H. P., and Patterson, E. L., *Fed. Proc.*, 1953, v12, 430.

22. Stokstad, E. L. R., *ibid.*, 1954, v13, 712.

23. Wachstein, M., and Meisel, E., PROC. SOC. EXP.

BIOL. AND MED., 1951, v77, 648.

24. Yang, C. S., and Olson, R. E., *Fed. Proc.*, 1954, v13, 482.

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Destruction of Virus Hemagglutination Inhibitor of Egg-White by Newcastle Disease Virus.* (21968)

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The viruses of mumps, influenza and Newcastle disease cause agglutination of certain mammalian red blood cells (RBC) by connecting these cells and forming a lattice-like matrix. If the ratio of virus and RBC is controlled, the reaction can be observed visually. In most instances the virus elutes or dissociates from the RBC after an indefinite time following mixing of the virus and red cells. The virus-treated cells are no longer capable of being "rebridged" by the virus but the virus retains its capacity to act again on untreated cells(1).

It is now known that normal ferret serum, serum from other animal species(2), a variety of tissue suspensions(3), ovalbumin(4), cow's milk(5), etc., will inhibit viral hemagglutination (HA). Inhibitor present in all of these substances seems to be very similar in composition to the "virus receptor" substance of the surface of erythrocytes which is commonly regarded as mucoprotein(6,7). Viruses of the mumps-Newcastle-influenza group can enzy-

matically destroy the inhibitor common to these materials. The exact role of this enzymatic action in infection has not been determined. One hypothesis is that firm adsorption to the cell surface is needed to allow the virus to enter the cell.

Since so many variations in characterized properties, *e.g.*, pathogenicity, heat stability of the hemagglutinin, of strains of Newcastle disease virus (NDV) exist, a study of the enzymatic activity of several strains of this virus seemed warranted. The following studies have utilized crude egg-white as the substrate or HA inhibitor in determining the mucinase activity of NDV.

Materials and methods. Viral strains. A repository for strains of NDV, maintained in the Department of Veterinary Science, University of Wisconsin, with the cooperation of the Agricultural Research Service, U. S. Department of Agriculture and the Newcastle Disease Technical Committee of the North Central States Region, has more than 80 strains, including 1 from Australia, 8 from Canada, 2 from Mexico, 1 from Japan, 8 from Europe and the remainder from the United States. All repository strains and the N strain of Dinter(8) were tested for their ability to destroy egg-white inhibitor (EWI). For the rate studies, however, only 7 strains were employed: Vic-Australia-1932, B1, Hert-Eng-1933, Milano-Italy-1945, GB-Texas-1948, (H)Najarian-Ohio-1948 and Roakin-NJ-1946. The Lee strain of influenza B virus was used as the indicator virus in the hemagglutination-inhibition (HI) tests. White Leghorn chick-

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en eggs from a commercial hatchery were used. As far as could be determined, these eggs came from flocks not vaccinated or infected with Newcastle disease.

Viral pools. Newcastle disease and N viruses were prepared by inoculating 10 or 11-day-old chicken embryos, intraallantoically, with 0.05 ml of a 10^{-2} dilution of stock, allantoic-fluid virus. Inoculated eggs were incubated at 98 to 99°F and candled 2 to 3 times daily. From embryos that had died after 24 hours, allantoic fluids were harvested and pooled. If the fluids were to be used within 4 to 5 days, they were stored at 4°C; otherwise at -20°C. The Lee strain of influenza B virus was prepared by inoculating 10 to 11 day-old, embryonated eggs, intraallantoically, with 0.05 ml of a 10^{-2} dilution of stock, allantoic-fluid virus. After further incubation at 98 to 99°F for 65 hours, the living embryos were chilled overnight and allantoic fluids harvested and handled as described for the other viruses. Calcium chloride (CaCl_2) 1 g, sodium chloride (NaCl) 9 g, boric acid (H_3BO_3) 1.203 g, and sodium tetraborate ($\text{Na}_2\text{B}_4\text{O}_7 \cdot \text{H}_2\text{O}$) 0.052 g were dissolved in 1 liter of distilled water, pH 7.0 ± 0.2 . *Hemagglutination tests.* Starting with a 1 to 5 dilution of allantoic-fluid virus, serial 2-fold dilutions were made in 0.25 ml volumes of physiological saline contained in 13 x 75 mm tubes. Chicken RBC, 0.25 ml of a 1% suspension in physiological saline, were thoroughly mixed with each dilution. The test was read after 30 to 45 minutes at room temperature. The endpoint, 1 HA unit, was the highest dilution of virus showing 1+ agglutination. One plus HA was not evident in most instances since the last tube to show agglutination was usually 4+. Consequently, most 1+ values were obtained by interpolation, e.g., if the highest dilution of virus showing agglutination was the 1 to 320 dilution and the degree of agglutination was 4+ the 1+ value was 320 plus three-fourths the difference between 320 and the next dilution, 640. The result is a value of 1 to 560 for 1+ agglutination.

Hemagglutination-inhibition test. Starting with a 1 to 100 dilution of EWI solution, serial 2-fold dilutions were made in 0.25 ml volumes of physiological saline contained in

13 x 75 mm tubes. Five HA units of indicator virus contained in 0.25 ml were mixed with each dilution. After 30 minutes at room temperature, 0.25 ml of a 1.0% suspension of chicken RBC was added to each tube and the HA patterns ascertained 45 minutes later. The endpoint was considered as the last tube to show complete inhibition of agglutination. *Egg-white inhibitor or ovomucin.* The "thick" albumin of fresh, White Leghorn eggs served as the source. Stock ovomucin was a 5% solution in calcium borate, buffered saline. Inhibitory activity was determined by HI testing using 5 HA units of heated Lee virus as the indicator antigen. One HI unit was considered as the smallest quantity of inhibitor required to completely inhibit the activity of 1 HA unit of the heated, Lee strain of virus. *Indicator virus.* Allantoic fluid from eggs infected with the Lee strain of influenza B virus was heated at 56°C for 30 minutes. This heated virus has the capacity to combine with inhibitor but not the ability to destroy it. Consequently, heated Lee virus serves well as a sensitive indicator for inhibitor titrations.

Results. In exploratory experiments employing 7 strains of NDV, each was effective in inactivating EWI substance. Subsequently, the entire collection of NDV strains in the repository and the N strain of Dinter(8) were tested for their ability to destroy EWI. Tests were conducted by adding 20 HA units of each virus to 1 ml of a 1:100 dilution of raw egg-white and incubating the mixture at 37°C for 15 hours. The reaction was terminated and virus activity eliminated by heating the mixture at 65°C for 30 minutes. The content of each tube was assayed for EWI content by an HI test employing heated, Lee strain of influenza B virus as the indicator. With each strain of NDV 20 HA units completely inactivated the inhibitor contained in 1 ml of 1:100 dilution of egg-white.

A series of experiments was designed to study certain kinetic features of the reaction between EWI and NDV. Strains Vic, Hert-E, Milano, GB, Roakin, Najarian and B1 were selected for the first experiment in which the rate of inactivation of 1280 HI units of EWI by each strain was determined as a function of its HA concentration. The reaction was car-

ried out at 37°C for 90 minutes after which time the reaction was stopped by heating as indicated. The content of each tube was assayed for residual EWI by HI testing with heated, Lee B indicator virus. Under the conditions of the experiments, the inactivation rates differed among strains and was dependent on the virus concentration within strains. For the Hert-E, Milano and B1 strains, only 32 HA units were required to completely destroy the inhibitor whereas 64 units of the Najarian and GB strains and 128 units of the Vic and Roakin strains were required.

The next experiment was to determine, as a function of time, the rate of inactivation of 1280 HI units of EWI by various quantities of 6 strains of NDV. The strains employed were the same ones used in previous experiments with the exception of the Najarian strain. Three quantities of each virus suspension were used, 256, 128 and 64 HA units. The virus and EWI mixtures were incubated at 37°C, and at 5 minute intervals for 30 minutes, a sample of each mixture was removed and heated at 65°C for 30 minutes. The residual content of EWI was assayed as in the previous experiment. The Milano, Hert-E and B1 strains not only inactivated the EWI most rapidly but behaved similarly, except for less activity with the 64 HA units concentrations with Hert-E. The inactivation rates of GB and Vic strains were similar but somewhat slower than of the Milano, Hert-E and B1. The Roakin strain did not inactivate any EWI within the 30 minute test period. In all cases where inactivation occurred, the rate of inactivation of EWI corresponded directly to the HA concentration of the virus.

The third experiment was designed to ascertain the rate of inactivation of EWI by strains of NDV as a function of EWI concentration. The strains of NDV were the same as employed in the previous experiment. Three concentrations of EWI, 1280, 640 and 320 HI units, were used and 128 HA units of each strain of virus were employed. The virus and EWI mixtures were treated exactly as described in the previous experiment. Hert-E, Milano and B1 gave similar inactivation patterns. The patterns of Vic and Roakin were similar while that of GB was intermediate be-

TABLE I. Capacity of NDV* in Allantoic Fluid Containing 0.4% Formalin to Destroy Egg-White Inhibitor.

NDV strain	Time in days after treatment with formalin					
	3	4	9	14	21	28
Vic	++†	—	—	—	—	—
Hert-E	++	++	++	++	++	±
Milano	++	++	++	++	++	—
GB	++	++	++	++	++	++
Roakin	++	++	++	—	—	—
Naj	++	++	++	++	++	++
B1	++	++	++	++	++	++
Md-II	++	++	++	++	—	—
Hert-M	++	++	++	++	++	±
Ky-50	++	++	++	++	++	++
Lederle	++	++	++	++	++	++
RO	++	++	++	++	++	—
Wagner	++	++	++	++	++	±
Appleton	++	++	++	++	++	++
CG	++	++	++	++	++	++
ViPol	++	++	++	++	++	++

* All strains of unformalinized NDV destroyed egg-white inhibitor after storage at 5°C for 25 days.

† —, ±, ++ = no, partial and complete destruction, respectively, of egg-white inhibitor in 1:100 dilution of egg-white.

tween the two groups. For all strains, the rate of inactivation was fastest with the 320 HI units of EWI. The rates of inactivation with 640 and 1280 HI units approximated each other but were slower than the 320 HI unit concentration.

The final experiment was concerned with the stability of the mucinase property of allantoic-fluid NDV when exposed to 0.4% formalin. Sixteen strains were employed. At 3, 4, 9, 14, 21 and 28 days after adding the formalin and storing the mixtures at 4°C, the ability of each strain to destroy EWI was tested. This was accomplished by adding 0.25 ml of formalinized, allantoic-fluid virus to a 1:100 dilution of ovomucin. After overnight incubation at 37°C, the mixtures were heated at 65°C for 30 minutes. The residual EWI determinations are recorded in Table I. All strains except Vic possessed mucinase activity at 4 days after formalinization and at 14 days Roakin had lost mucinase activity. The Md-II strain did not inactivate egg-white inhibitor after 21 days. After 28 days, RO was inactive, while Hert-E, Hert-M and Wagner were only partially active.

Discussion. The mucinase activity of the mumps-Newcastle-influenza group of viruses is

widely recognized. If viral mucinase has any biological significance, it remains to be defined. Many hypotheses have been proposed but none have been definitely proved. However, it is well established that viruses are intracellular parasites which must invade and enter a susceptible host cell before multiplication can occur. This immediately raises the question of the mechanism of penetration of the host cell and the relation of inhibitors to the infectious process.

Almost all cells of the body, especially those of the respiratory tract, and except those of the skin, are protected by a mucous secretion. Since the viruses mentioned above have, in the main, an affinity for respiratory tract tissues, mucinase may have as its function the destruction of this protective material in order to clear the way for the attachment of the virus to the host cell preparatory to its penetration.

Strains of NDV are known to vary markedly from one another in properties such as pathogenicity, heat stability of the hemagglutinin and neurotoxicity. For some strains these properties have been well defined. However, no one has reported any mucinase studies of the various strains of NDV with the purpose of possibly relating them to some of the known properties. This purpose prompted, in part, the studies presented in this paper.

All strains of the repository were able to destroy the hemagglutination inhibitor of hen's egg-white. After the preliminary experiment, 7 strains of NDV markedly different from each other were selected for determination of the rate of the reaction. Despite the fact that these studies were performed in a crude fashion since neither the enzyme nor the substrate were separated and purified from their native sources, the results obtained appear susceptible to logical explanation. The first concern was with the rate of inactivation of EWI as a function of virus concentration. The 7 strains employed fell into 3 groups: (1) Hert-E, Milano and B1; (2) Najarian and GB; (3) Vic and Roakin. Group 1 inactivated the inhibitor rather quickly while the other 2 were progressively less active. With the exception of the Najarian virus, these 7 strains were

used in other studies of inactivation rate as follows: (1) Rate of inactivation of 1280 HI units of EWI by various quantities of NDV as a function of EWI concentration. For each study, the 6 strains fell into 3 groups which are the same groups as described for the initial rate study. The greater the quantity of virus (enzyme), the faster was the inactivation rate. For the inhibitor concentration studies, the results were slightly different since the 320 HI units were inactivated in all cases at a faster rate than the 640 and 1280 units, which were inactivated at about the same rate. Whether the slower rate for the larger concentrations of the inhibitor was due to higher viscosity of the crude substrate solution is not known. There was also observed in all of these experiments a lag period whose duration depended on the concentration of the enzyme.

The activity groups into which these strains fell appear subject to correlation with only one known property *i.e.*, the rate of their elution from red cells. There is no apparent correlation between mucinase activity and pathogenicity for chickens since 2 highly pathogenic strains, Hert-E and Milano and a non-pathogenic strain, V1 constituted the group with the greatest mucinase activity.

A study dealing with the stability of mucinase to 0.4% formalin indicated this enzymatic activity was fairly stable.

Summary. Studies were made on the presence and mucinase activity of Newcastle disease virus and of the stability of this enzyme function to formalin. (1) All strains of Newcastle disease virus studied possessed mucinase activity although the rates at which they inactivated egg-white inhibitor differed greatly. (2) The rate at which egg-white inhibitor was inactivated was dependent on enzyme (virus) concentration. The concentration of egg-white inhibitor also affected the rate of its inactivation. (3) The rate of inactivation by Newcastle disease strains of virus could be correlated only with one known property—its rate of elution from red blood cells. (4) Newcastle disease virus mucinase was fairly stable to formalin.

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1. Hirst, G. K., *J. Exp. Med.*, 1942, v76, 195.
2. ———, *ibid.*, 1942, v75, 49.
3. Friedwald, W. F., Miller, E. S., and Whatley, L. R., *ibid.*, 1947, v86, 65.
4. Lanni, F., and Beard, J. W., *Proc. Soc. Exp. Biol. and Med.*, 1948, v68, 312.
5. Lanni, F., Sharp, D. G., Eckert, E. A., Dillon,

E. S., Beard, D., and Beard, J. W., *ibid.*, 1949, v72, 227.

6. McCrea, J. F., *Austr. J. Exp. Biol. Med. Sci.*, 1948, v26, 355.

7. Gottschalk, A., and Lind, P., *Brit. J. Exp. Path.*, 1949, v30, 85.

8. Dinter, Z., *Tierärztliche Umschau*, 1949, p. 229.

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Importance of Optimum Amounts of Virus to Demonstrate Neutralizing Antibody Rise in Convalescent Poliomyelitis Sera.* (21969)

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(Introduced by L. Emmett Holt, Jr.)

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A study of 38 patients infected with poliomyelitis viruses showed a 5-fold or greater increase in the homologous antibody titer in 35 (92%). This finding was at variance with that of other workers(1,2), who postulated that the rise in neutralizing antibodies occurred so rapidly that often no increases in titer were demonstrable. Thus Miller and Wenner, using a virus concentration of 32 TCID₅₀[†] in their neutralization tests, observed a 10-fold rise in homotypic antibody in only 3 of 14 patients(2). Tests for homologous antibody were carried out in only 7 patients, none of whom showed a similar rise. Melnick and his associates, with a virus inoculum of 100 TCID₅₀, reported rises of homotypic antibody in 15 (37%) of 41 patients from whom poliomyelitis viruses had been isolated(1). Homologous antibody formation was not studied by these workers.

This report presents evidence that the demonstration of increases of homologous antibody in patients infected with poliomyelitis viruses is related to the dose of virus used in the neutralization test.

Materials and methods. Data are derived

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[†] TCID₅₀—Tissue culture infective doses, 50% end point.

from 38 patients studied in New York City in 1953, a non-epidemic year.[‡] Their stools were collected within 2 weeks of onset of illness and after concentration in the Spinco high speed centrifuge, yielded poliomyelitis viruses as follows: Type 1, 25 patients; Type 2, 2 patients; Type 3, 11 patients. All but 4 showed some degree of weakness or paralysis. Serum specimens were collected at frequent intervals and tested for neutralizing antibodies against the patient's own virus and the 3 prototypes. To be reported here are the results of tests carried out on mixtures of homologous virus and serum obtained usually in the 1st or 2nd week of illness, in the 3rd or 4th week, and lastly at about the 12th week. Trypsinized monkey kidney cells grown in tissue culture in glass tubes[§] and examined microscopically for cytopathogenic changes constituted the test object for isolation of virus and for neutralization tests. Neutralization tests were carried out usually with serial 4-fold dilutions of serum, inactivated at 56°C for 30 min. Serum dilutions were mixed with an equal vol-

[‡] We are grateful to the staffs of Willard Parker, Mt. Sinai, Babies', and Flower-5th Avenue Hospitals for supplying material for this investigation.

[§] For the past year tubes containing cultures of monkey kidney cells supplied by Microbiological Associates, Inc., Bethesda, Md., have been used.

TABLE I. Relation of Increase in Neutralizing Antibody and Dose of Virus Used in Test. 38 patients.

Day of illness 1st serum obt.	Virus type in stool	Dose of virus in test*	Antibody fold-increase
7	1	3.2	500
6	3	2.7	400
5	3	4.0	200
4	2	2.3	200
2	1	2.7	200
11	1	2.5	60
8	3	3.7	50
7	1	4.2	40
11	1	3.7	40
3	1	3.0	30
7	1	3.5	25
8	1	2.7	25
18	3	3.9	25
7	3	1.2	25
8	1	3.7	16+?
5	3	3.2	16
10	1	3.5	16
7	1	4.5	12+?
7	1	1.5	12+?
8	1	4.5	12
8	3	2.5	12
3	1	3.2	12
8	1	2.0	10+?
9	3	4.2	10
5	3	4.0	10
4	1	4.0	10
6	3	3.0	10
4	1	2.5	10
14	2	1.0	10
8	1	2.7	7
9	1	4.7	7
18	1	2.7	6
18	1	3.2	5
9	1	2.5	5
16	1	3.5	5
7	1	4.0	3
14	3	2.7	2
10	1	4.9, 3.5, 2.4, 1.2	0

* Expressed as negative log. † 92% of patients.

time of a selected dilution, usually 1:500, of the patient's virus. The latter was titrated simultaneously in each test against normal monkey serum. The mixtures were incubated at room temperature for one hour. 0.1 cc of each dilution was pipetted into tissue culture tubes containing 0.9 cc of standard medium.¶ As a

¶ The "standard medium" consisted of Earle's balanced salt solution, lactalbumin hydrolysate 0.5% and calf serum, 2%. 50 units of penicillin and 50 mg of streptomycin per ml of medium were added. Recently 20 units of mycostatin (Squibb) per ml were added which has helped considerably to suppress the growth of yeasts.

rule 3 tubes per dilution were used. In some of the "box" titrations 2-fold dilutions of serum were mixed with decimal dilutions of virus and inoculated into 6 tubes per dilution. All tubes were examined daily under the microscope for cytopathogenic effects. The seventh day reading was taken as final. Antibody titers were calculated by the method of Reed and Muench and expressed as the 50% serum dilution endpoint (log of reciprocal).

Results. Table I shows the relation of degree of antibody rise and dose of virus used in the test. A 5-fold or greater increase in homologous antibody obtained in 35 (92%) of 38 patients. Twenty-nine patients (76%) had antibody rises from 10 to 500-fold. In 6 patients (16%) increases from 5 to 7-fold were obtained. It is evident that antibody increments as great as 200-fold were obtained with as much as 4 logs of virus. The average amount of virus used in all the tests was about 3 logs. Substantial increases in antibody were demonstrated even when the first serum specimen was obtained as late as the second or third week of illness. In the group whose antibody rises were under 10-fold the first serum was obtained on an average of the 12th day of illness, whereas in the group of 29 patients whose antibody rises were 10-fold or greater, the first serum was drawn on an average of 7.2 days after onset. If the reverse calculation is made, it will be seen that the average antibody rise is 95-fold in patients whose first serum was obtained in the first 7 days of illness as compared with an average of 16-fold for those whose first serum was obtained between the 8th and 18th days.

"Box" Neutralization Tests with Homologous Virus. During the beginning of this investigation in most of the individual tests a single relatively large amount of virus was used against serial dilutions of serum. It seemed possible that the greater number of definite antibody rises observed might be related to the larger doses of virus employed as compared with those used by previous observers. This possibility was investigated by simultaneous titrations of virus and serum (so-called "box" titration) from 3 patients—2 infected with Type 1 (A and B) and one with Type 3 (C). The results ap-

TABLE II. "Box" Neutralization Tests.

Patient	Day of illness serum drawn	A										B																		
		Logs of homologous virus in test					Antibody titer (log of dilution)					Logs of homotypic virus in test					Logs of homologous virus in test													
		.5	.6	.7	1.5	1.6	1.7	2.5	.6, 1.2	2.2	2.3	2.6, 2.4	.6	1.1	1.7	4.5	4.6	4.7	5.6	.4	.6	1.4	1.6	2.4	2.6	3.4	3.6	4.4	4.6	5.4
A	3	3.4+	?																											
	80	3.5			2.8																									
B	9		3.2+	?																										
	82		3.6		3.4			1.6																						
C	15			2.6+				2.2																						
	93			3.2				2.2																						
Fold increase	0	2-?	?	?	3	16	4	100, 16	16	3	12	50	12	1.1																

* 6 tissue culture tubes per dilution of serum.

+ Idem

; serial 2-fold dilutions of serum.

pear in Table II (Section A). It is evident that little or no antibody rise was demonstrable with the smallest doses of virus (.5, .6, .7 log). With 1 log higher dose only 1 patient (B) showed a significant rise; with 2 logs, 2 patients, and with 3 logs, all 3 patients showed substantial rises. When the sera were "overwhelmed" with 4.5-5.6 logs of virus, no significant antibody increase resulted. In the test with patient B's virus and serum the accuracy was enhanced by using 6 tubes per dilution. In the case of patient C, a 12-fold antibody increase was the maximum achieved. This occurred with 3.7 logs of virus. It may be that this relatively small antibody rise is accounted for by the lateness of obtaining the acute phase serum, e.g., 15th day of illness. In any event, the result of this test shows a trend in keeping with the pattern of the other two.

"Box" Neutralization Tests with Homotypic Virus. It was important to learn if this apparent relation between antibody rise and dose of virus would apply to homotypic as well as to homologous virus. Similar "box" titrations were performed with the sera of 2 patients, B and C, mixed with Type 1 (Mahoney) and Type 3 (Leon) viruses, respectively. To increase the accuracy of the tests, these box titrations were carried out with serial 2-fold dilutions of serum against decimal dilutions of virus and the mixtures were inoculated into 6 tissue culture tubes per dilution. The results are presented in Table II (Section B). As with homologous viruses, little or no antibody rise resulted with the smallest doses of homotypic virus. With 3.4 and 3.6 logs, 10-fold or higher increments were obtained. Unlike the other tests in which the largest doses of virus either obliterated or diminished the difference in antibody level between the two sera, in the case of patient C's sera and the heaviest concentration of Leon virus (4.6 logs), a 10-fold increase was still demonstrable. Then actual titers are not very different, however, from those obtained with C serum and homologous virus (Section A). In that test when a comparable dosage (4.7 logs) of C virus was used, even though it seemed to result in a 4-fold antibody rise, the titers of each serum (0.6 and 1.1) did not differ significantly from those obtained with Leon virus (0.5 and 1.5).

From the results of these 2 tests with homotypic viruses, it would appear that the relation of antibody increase to virus dose was not limited to the patient's own virus, but a similar pattern was obtained with homotypic viruses, Mahoney and Leon, respectively.

Discussion. The results obtained in individual tests with various virus strains and sera indicated a relationship between increase in neutralizing antibody and concentration of virus used in the test. This was confirmed by "box" neutralization tests in which virus and 2 serum specimens from the same patient were titrated simultaneously. In the zones of antibody excess and virus excess, respectively, there was little or no apparent increase in antibody from acute to convalescent phase of illness. In the middle dosage range of about 3 logs of virus regular antibody increases were demonstrable. The explanation of this phenomenon is not clear. The question arises as to whether antibodies found in the acute stage differ qualitatively or quantitatively from those found in convalescence. Work is being done currently on the dynamics of virus-antibody union which may shed light on the problem. For example, is the union between virus and "late" antibody more stable than that which occurs with "early" antibody? Are there detectable differences in the velocity of the reaction? Does "late" antibody have a greater avidity for virus than "early" antibody? The results of the present report have been obtained entirely with the use of cultures of monkey kidney cells as the test object of virus activity. It is important that Steigman and Sabin(3) and Winsser and Sabin(4), who carried out quantitative neutralization tests in monkeys, observed substantial homologous antibody increases between 16 and 32-fold with amounts of virus ranging from 160 to 1000 PD₅₀. The results of the present study indicate that the relation between antibody rise and virus dose is not limited to homologous systems. The acute and convalescent sera of 2 patients (one with a Type 1 infection, the other Type 3) showed similar antibody trends against the homotypic virus, Mahoney and Leon, respectively.

Even though the mechanism by which it works is not understood at present, the phenomenon appears to have important practical as well as fundamental implications. The neutralization test may still prove to be an important diagnostic tool provided the dose of virus is properly adjusted. It would appear that approximately 1000 TCID₅₀ is the optimum dose of virus. Another factor of importance in demonstrating antibody increase is the time of drawing the "acute" serum sample—the earlier the better. The study of basic problems concerned with the kinetics of the poliomyelitis virus-antibody reaction will be advanced by the use of Dulbecco's new plaque method(5).

Summary. The formation of neutralizing antibodies against their own virus was studied in 38 poliomyelitis patients. In the sera of 35 (92%), 5-fold or greater antibody increases were demonstrated. Twenty-nine (76%) exhibited rises between 10 and 500-fold. The average dose of virus used in separate tests was about 3 log dilutions. "Box" neutralization tests revealed that little or no antibody increase was demonstrable in the zones of antibody or virus excess. Substantial antibody rises were obtained in the middle zones, when between 2.5 and 4.6 log dilutions of virus were used. A similar trend of antibody increase was found with homotypic virus studied in two individuals. These results seem to indicate a relation between increase in neutralizing antibody and concentration of virus in the test. The mechanism by which this phenomenon operates is not understood but it appears to have both fundamental and practical implications.

1. Melnick, J. L., Ramos-Alvarez, M., Black, F. L., Girardi, A. J., and Nagaki, D., *Yale J. Biol. and Med.*, 1954, v26, 465.

2. Miller, C. A., and Wenner, H. A., *Pediatrics*, 1954, v14, 573.

3. Steigman, A. J., and Sabin, A. B., *J. Exp. Med.*, 1949, v90, 349.

4. Winsser, J., and Sabin, A. B., *ibid.*, 1952, v96, 477.

5. Dulbecco, R., and Vogt, M., *ibid.*, 1954, v99, 167.

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Effect of Levallorphan and Nalorphine upon Barbiturate-Induced Respiratory Depression in Rats.* (21970)

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N-allylnormorphine HCl, (nalorphine), and levo-3, hydroxy N-allylmorphinan tartrate, (levallorphan), have been shown to be effective antagonists of analgetic-induced respiratory depression both clinically and experimentally (1-9). However, with respect to the possible use of nalorphine against barbiturate depression the available reports are confusing, for some authors report that nalorphine is an effective barbiturate antagonist (10-12), while others found it to be ineffective (3,13-15). No reports are available concerning the effectiveness of levallorphan as an antagonist to barbiturate depression.

This study was undertaken to determine in rats the effectiveness of both nalorphine and levallorphan as antagonists to the respiratory depressant effects of some of the more commonly used barbiturates.

Procedure. Male albino rats (Sprague-Dawley) 200-300 g in weight were used. All experiments were acute, each animal serving as its own control, and about 50 experiments were carried out. The procedure was similar to that described previously (7), and consisted of cannulation of the trachea under ether, and infiltrating the area with a local anesthetic. The animal was restrained and connected to a one-way valve system to measure tidal air. After a control period, the barbiturate under examination was given, in a dose determined previously to produce 50% respiratory depression, then either nalorphine or levallorphan were administered by intraperitoneal injection

at various dosage levels. The barbiturates examined were phenobarbital, barbital, amytal, pentobarbital and secobarbital.

Results. Levallorphan exerted no demonstrable effect in reducing the barbiturate-induced depression of respiration when used in dosage ratios of 1:1 or less of levallorphan to barbiturate. Because of this ineffectiveness of levallorphan a second series of experiments was carried out in the dose of the antagonist was increased. Fig. 1. Levallorphan was effective in diminishing only the pentobarbital-induced respiratory depression, and only when twice as much of the antagonist as depressant was employed. No noticeable stimulatory effects were observed with other barbiturates.

In the experiments with nalorphine only the higher dosage levels were tested, these were identical with doses used in the second series with levallorphan. Again, as with levallorphan, nalorphine was only effective in diminishing pentobarbital-induced respiratory depression, and only when twice as much nalorphine as pentobarbital was used.

Discussion. Both levallorphan and nalorphine were found to be moderately antagonistic to pentobarbital-induced depression but only when very large doses of these 'antagonists' were employed. On the other hand, no stimulatory effects were observed when these 2 compounds were used with phenobarbital, barbital, amytal and secobarbital. This is in marked contrast to the effect of levallorphan and nalorphine upon respiratory depression induced by the narcotic analgetic compounds, where the effective ratio of antagonist to depressant ranged from 1:1 to 1:30 for Dromoran, methadone, morphine, alphaprodine and meperidine. For example, the ratios in the case of morphine sulfate depression are 1:6.7, and 1:5, and in meperidine depression 1:30 and 1:5 for levallorphan and nalorphine respectively (7).

In this present series of experiments levallorphan

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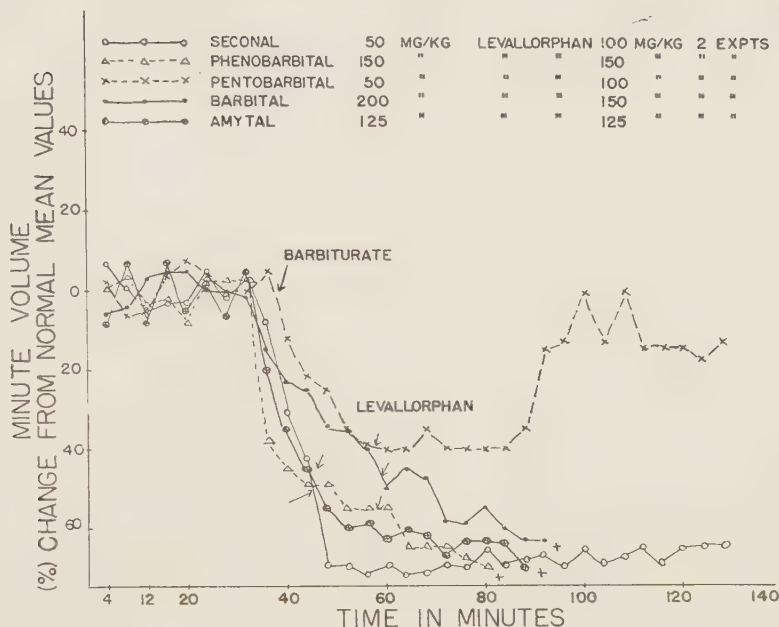


FIG. 1. Effect of levallorphan upon respiratory depression produced by various barbiturates. Effect with nalorphine was essentially the same, and the dosages were identical.

lorphan and nalorphine were not used in doses greater than 150 mg/kg because this would have exceeded the LD_{50} in rats for both compounds, (approximately 180 mg/kg for both levallorphan and nalorphine).

Eckenhoff *et al.*(13) found that nalorphine was ineffective in combating clinical barbiturate depression. However, Belford and Kao (11) and Vivante *et al.*(12) later reported that nalorphine was effective in relieving pentobarbital-induced respiratory depression in the dog. Clinically, Dulfano *et al.*(10) found nalorphine to be effective in combating barbiturate depression in several instances. On the other hand, several recent reports(3,14,15), have pointed out the failure of nalorphine, both experimentally and in man, to relieve respiratory depression from barbiturates.

There are no reports in the literature concerning the ability of levallorphan to combat respiratory depression produced by barbiturates. From the present study it would appear that both levallorphan and nalorphine can restore partially the respiratory minute volume in rats when it has been depressed by pentobarbital. However, this effect could only be achieved with very large doses.

Summary. It was observed that nalorphine and levallorphan were ineffective in antagonizing the respiratory depression produced in rats by large doses of phenobarbital, barbiturates, amytal and seconal. The two compounds did, in large doses, cause some diminution in the depression produced by pentobarbital.

1. Lasagna, L., *A.M.A. Arch. Int. Med.*, 1954, v94, 532.
2. Winter, C. A., Orahovats, P. D., Flataker, L., Lehman, E. G., and Lehman, J. T., *J.P.E.T.*, 1954, v111, 152.
3. Salomon, A., Marcus, P. S., Herschfus, J. A., and Segal, J. S., *Am. J. Med.*, 1954, v17, 214.
4. Benson, W. M., O'Gara, E., and Van Winkle, S., *J.P.E.T.*, 1952, v106, 373.
5. Gross, E. G., and Hamilton, W. K., *J. Lab. Clin. Med.*, 1954, v43, 938.
6. Foldes, F. F., Swederlow, M., and Siker, E. S., *J.P.E.T.*, 1955, v113, 21.
7. Costa, P. J., and Bonnycastle, D. D., *ibid.*, 1955, v113, 310.
8. Eckenhoff, J. E., Helrich, M., Hege, M. J. D., and Jones, R. E., *ibid.*, 1955, v113, 332.
9. Thomas, D. V., and Tenney, S. M., *ibid.*, 1955, v113, 250.
10. Dulfano, M. J., Mack, F. X., and Segal, M. S.,

New Eng. J. Med., 1953, v248, 931.

11. Belford, J., and Kao, F. F., *Fed. Proc.*, 1954, v13, 336.

12. Vivante, A., Kao, F. F., and Belford, J., *J.P.E.T.*, 1954, v111, 436.

13. Eckenhoff, J. E., Elder, J. D., and King, B. D.,

Am. J. Med. Sc., 1952, v223, 191.

14. Gruber, C. M., Jr., *J.P.E.T.*, 1954, v111, 409.

15. Boyd, E. M., and Pearl, M., *C.M.A.J.*, 1955, v73, 35.

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Morphology of Characteristic Particles Associated with Avian Erythroblastosis.* (1971)

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Erythroblastosis and myeloblastosis (granuloblastosis)(1) are highly malignant leukemic diseases of viral etiology in the chicken. In the latter condition involvement of the precursors of the myelocytes(2) results in the occurrence in the blood circulation of very large numbers of primitive cells of myeloblastic features. The virus of erythroblastosis presumably affects the progenitors of the red blood cells and the disease is characterized(3) by the presence of many erythroblasts in the circulating blood. That the respective viruses, likewise, are present in the circulation in both conditions is attested by transmissibility of the diseases with filtered blood plasma. By application of ultracentrifugal procedures, the virus of myeloblastosis has been obtained from the plasma of diseased chicks in appreciable amounts in purified preparations for physical, chemical and biological characterization(4,5). The findings with myeloblastosis have stimulated the undertaking of similar studies with the virus of erythroblastosis. Examination of filtered blood plasma from chicks with this disease has revealed characteristic particles, which by virtue of the experience with myeloblastosis, may represent the etiological agent of erythroblastosis. This paper describes results of preliminary investigations on the morphology of these particles observed

in electronmicrographs.

Materials and methods. Erythroblastosis virus was obtained from Dr. Astrid Fagraeus of the State Bacteriological Laboratory, Stockholm, who had received it previously from Dr. J. Engelbreth-Holm at the Department of Pathological Anatomy, University of Copenhagen. The agent has been passed in this country by means of filtered plasma injected whole, or diluted, in 0.1 ml volumes in 3-day to 3-month-old chickens. The birds employed routinely were inbred White Leghorns of line 15 developed(6) at the Regional Poultry Research Laboratory, East Lansing, Mich. and the same as those used in the work with myeloblastosis(7). As will be developed in later reports, the strain of erythroblastosis appears to be a characteristic entity entirely distinct from myeloblastosis with respect both to the pathological manifestations and to host-response to the agent. Small chicks with the disease were bled by section of the sagittal sinus and larger ones by heart puncture. The blood was received in chilled tubes containing heparin and cleared of cells by 2 cycles of low speed centrifugation. The plasma was recovered in each case with fine tipped pipettes, then filtered with celite and passed through Selas filters by the technics(8) employed in the work with myeloblastosis. Donor birds were selected on the basis of the number of erythroblasts seen in routine daily blood smears, and bleeding was effected only when the chickens were near death. Once the chicks became obviously ill, as indicated by lassitude or weakness, the condition pro-

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gressed rapidly to death within a few hours. In most instances the chickens were closely watched, and bled when they became moribund. During these stages of the disease, the increase in erythroblasts was rapid, and it seemed likely that the virus content of the plasma might, likewise, reach the highest levels in the few hours before death of the host. Thus far, none of the plasmas has exhibited significant activity to dephosphorylate adenosine triphosphate in the microscreening test (9). Consequently, this criterion of plasma content of virus, so useful in the study of myeloblastosis, has not been available in the experiments with erythroblastosis. However, in contrast with host response to the virus of myeloblastosis(10), 2-month-old chickens were not greatly different in susceptibility than 3-day-old birds to infection with erythroblastosis virus. This was a most important factor, since the older birds furnished much larger volumes of plasma as source of the virus. The ages of the donor chicks used in the present work were from 3 to 60 days. Blood was drawn 7 to 15 days after inoculation of the virus. The technics employed for examination of the particles in erythroblastosis with the electronmicroscope were identical with those applied in the studies on the virus of myeloblastosis(8). Although observations were made with the particles in plasma, the principal investigations involved the particles in concentrates obtained by 1 or 2 cycles of sedimentation. The best results were obtained with individual plasmas spun, undiluted, in collodion tubes of appropriate volume. The particles were concentrated by centrifugation at 16,400 x g for 60 minutes and resuspended in small volumes, 5 to 25% of those of the initial plasmas, in normal saline solution containing 0.001 M phosphate of pH 7.0. These concentrates from individual plasmas or, more often, in pools (pooled only after the initial concentrating procedure) were centrifuged at 7,000 x g for 5 minutes to eliminate aggregated material. In some cases the procedures of high and low speed centrifugation were repeated for a second time. Preparations were made for electronmicrography either with a drop of undiluted concentrate dried on an agar surface, or the particles

were spun down on agar(8) from suspensions diluted 1-10 to 1-100. The particles were fixed on the agar surface with osmic acid vapor and examined either after shadowing with chromium or without shadowing. Examinations were made with comparable technics of the particles directly in plasma. Counts of the particles in plasma or concentrates were made by sedimentation on agar in the usual way(8).

Results. The virus of erythroblastosis is relatively highly "virulent" in the sense that the chicks of the inbred line 15 were very susceptible to infection. With the largest doses of the agent, 0.1 ml of the best plasmas, onset of the disease, marked by the appearance of polychrome erythrocytes(3), was as early as 48 hours. Death followed frequently within 4 to 5 days. This appearance of intensity of host-virus interaction occurring in the induction and progression of the disease, as compared with the process in myeloblastosis, is emphasized by the observation of relatively low concentrations of characteristic particles which might represent virus in the plasmas.

That low particle content of the plasma is characteristic of erythroblastosis has been evident from several indications. In no case has the plasma exhibited visible turbidity ascribable to the presence of particles in high concentration. Ultracentrifugation results in no visible or very small pellets. In most instances material concentrated 3 or more times with respect to the volume of the starting plasma showed little opalescence, though occasional plasmas yielded quite turbid suspensions at

TABLE I. Approximate Characteristic Particle Content of Various Individual Plasmas from Chicks with Erythroblastosis Counted Either Directly in Plasma or Estimated from Counts with Concentrates of the Agent.

Donor	Age at bleeding (days)	Interval since inoculation (days)	Erythroblasts in blood smears†	Particles/ml plasma, $\times 10^8$
O 913*	17	12	4	257
O 911*	15	10	5	6.6
N 62	17	12	3	117
O 969	17	12	4	47
N 98	19	9	8	3.3
N 225	19	9	6	27

* Estimated from counts on concentrates.

† No. of cells/oil immersion field.

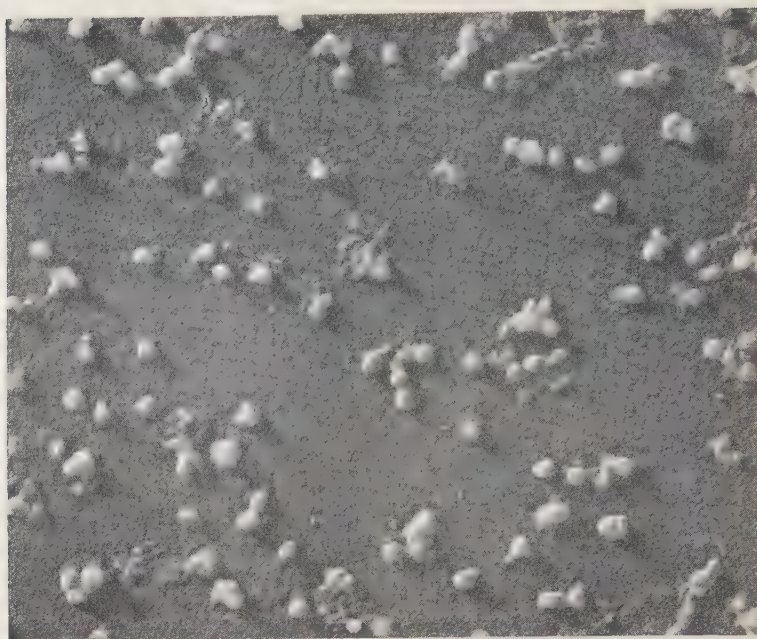


FIG. 1. Electronmicrograph of particles from the plasma of chick with avian erythroblastosis obtained by sedimenting the particles on an agar surface. Particles were shadowed with chromium. Magnification 27,600 \times .

20 times concentration. Finally, approximate estimates obtained by counting the particles in concentrates, after unmeasurable loss in the sedimentation procedure, as well as more accurate determinations made by counting the particles directly in plasma showed highly variable results ranging (Table I) from about 10^8 to 10^{10} particles per ml of plasma. The data of Table I are cited as representative of the findings. Many plasmas and concentrates were examined, but counting was excluded in most instances because of aggregation or excess amorphous material extraneous to the particles in relation to the very small numbers of particles.

Despite the technical difficulties, micrographs have been obtained clearly revealing the characters of the particles. One of the best pictures is that of Fig. 1 obtained with the concentrate of plasma O 913, Table I. The particles were sedimented from 4 ml of the plasma and taken up in 1 ml of saline. After low speed centrifugation the particle content of this suspension was 1.03×10^{11} per ml. The suspension was diluted 1-100 (equivalent to a 1-25 dilution of the original plasma), and the particles were spun onto agar.

The electronmicrograph reveals a population of particles of considerable homogeneity of kind which are of variable size and, in general, of spheroidal shape. There is present, also, amorphous material obviously different from the predominant particles and relatively few particles of a different magnitude of size from that of predominant ones. Measurements on small numbers of the particles of various sizes gave the value 102 $m\mu$ as the average diameter. It is seen that the particles are not regularly rounded but exhibit surface depressions, folds and protrusions from the main body of the particles, some as short tails or flattened membrane-like structures. The general characters of the particles are clearly shown, though the preparation contained much other material.

Shadowing with metal, though increasing the contrast and revealing 3-dimensional features of virus and other particles, obscures completely all except occasional contour evidence of internal structure. Pictures of unshadowed particles are difficult to obtain, but an informative example was available in the present work. A small drop of a suspension of twice sedimented particles was placed di-

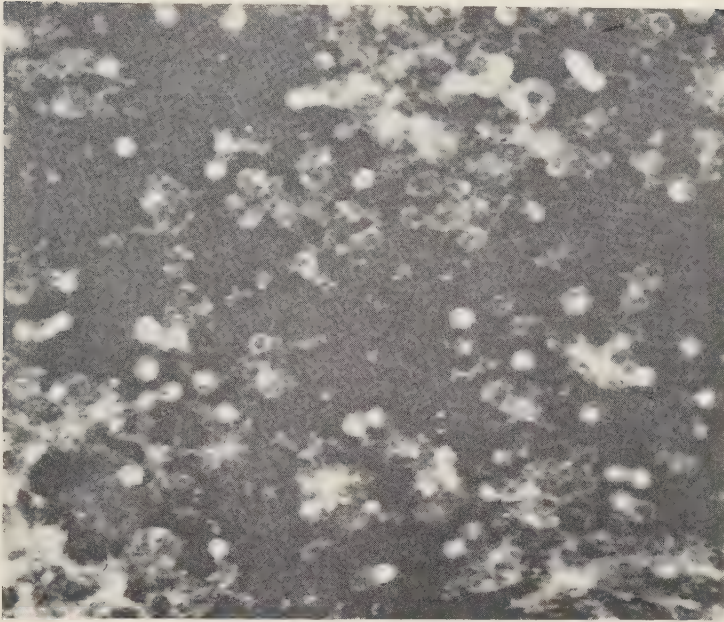


FIG. 2. Electronmicrograph of particles from the plasma of chick with avian erythroblastosis obtained by drying a drop of concentrate on an agar surface. Preparation was not shadowed. Magnification 34000 \times .

rectly on agar, dried, fixed with osmic acid and examined without shadowing. Fig. 2 shows 2 principal types of structures. One is represented by an essentially circular image with a small center of material of high electron absorbing power surrounded by a "membrane-like" substance of very low contrast. Similar images have been seen(11) with the virus of myeloblastosis, which is a particle of very low hydrated density(12) and is regarded as consisting primarily of watery, gel-like material surrounding a small region, or regions, of concentrated, relatively dense (to electrons) internal material. The images of the particles in erythroblastosis have the same appearance.

The second structures of interest are those having the appearance of empty, low contrast membranes greatly resembling particle "ghosts" consisting of the material surrounding the internal masses of the other images. In many instances there are seen empty spaces or apparent rents in the substance seeming to indicate space previously occupied by the dense material or through which it escaped. Other images have the appearance of fragments of the low-contrast material. Such

images were seen not infrequently in the present studies, but the mechanism for the production of the material, that is, for the rupture of the particles and the escape of the dense constituent is not known. Although the interpretation may be faulty in detail, it seems certain that these structures of low contrast must be related to the intact particles, and do not resemble any of the usual nonspecific components of plasma either from normal birds or chicks with erythroblastosis. It is especially notable that many of the low contrast images are fairly regular in outline, are of the size of the images of the intact particles, and have the same appearance as that of the peripheral material of the specific particles.

Discussion. The present findings do not suffice to establish unequivocally the biological nature of the particles described. The component has not been observed, however, in the plasmas of normal chicks, and it occurs as a population of particles of considerable homogeneity with respect to kind only in diseased individuals. The characters of the particles and their obvious relation to the occurrence of erythroblastosis, together with considerable experience in the study of the

contents of chicken plasma in investigations of myeloblastic leukemia, constitute strong presumptive evidence that the particles represent the etiological agent of the disease.

The particles in erythroblastosis are remarkably similar to those of the virus of myeloblastosis. In the case of the latter agent, a variety of investigations has indicated that the myeloblastic leukemia virus is a spheroidal particle of low hydrated density and high water content. Most of the volume of the virus is constituted of material of low electron absorbing properties surrounding a small mass of electron-dense material, which may be regarded as nucleoprotein as in the case of analogous structures in other viruses. All of the evidence obtained in the present work is compatible with the view that the characteristic particles in erythroblastosis are essentially identical in physical structure with the agent of myeloblastosis. It would be fruitless to extend the comparison, inasmuch as sufficient amounts of the particles are accessible for analyses by other physical methods. It is desirable to note that the size value for the particles in erythroblastosis, 102 $m\mu$, is somewhat smaller than that, 120 $m\mu$ for the agent of myeloblastosis. This difference, however, is not regarded as significant. A real difference between the two diseases is the particle content of the plasma. In erythroblastosis, the concentration of particles reaches levels of approximately 10^{10} per ml, whereas in myeloblastosis the levels(8) are as high as 2×10^{12} per ml. Another striking difference between the diseases resides in the lack of adenosine triphosphatase activity in the micro test in erythroblastosis. This does not signify a lack of enzyme activity of this virus, since the results may well have been related principally to the low concentration of the particles in the individual plasmas.

Of particular interest is the bearing of the present findings on the general problem of the etiological interrelationships among the various diseases classified(1) as members of the leukemia complex. These relationships can be determined only by intensive studies of the physical, chemical and biological properties of the agent associated with each condition. A considerable basis of information has been es-

tablished with the agent of myeloblastosis(4). It would appear that the superficial physical aspects of the morphology of the particles in erythroblastosis are indistinguishable from those of the agent of myeloblastosis. Within the limits of the small number of examinations thus far made, the agent of a third form of leukemia(13), the RPL strain 12 of lymphomatosis, does not differ appreciably in morphology from the particles in erythroblastosis and myeloblastosis. This resemblance, however, may be entirely superficial, and further study may reveal significant physical and chemical differences. It would be well to emphasize the biological specificity of these 3 agents in the cell type involved; in the pathological manifestations; and in the character of host response in the induction of the respective diseases. These investigations on the agent of leukemia have been of principal value thus far in the physical demonstration of the virus entities in the plasma of birds with myeloblastosis and characteristic particles which are probably the etiological agents of erythroblastosis and lymphomatosis and of the feasibility of obtaining the particles in the state and amount needed for critical experimentation.

Summary. A particulate component, which may be the etiological virus, was isolated by ultracentrifugation of the blood plasma of chickens with erythroblastosis. The particles were of spheroidal shape and variable size, averaging approximately 102 $m\mu$ diameter. Micrographs of unshadowed preparations showed circular images of an appearance suggesting that the particles consisted predominantly of a watery, low-electron contrast, gel-like material surrounding a small relatively dense internal structure. The findings were compared with those obtained in the analogous studies of particles representing the virus of myeloblastosis and that of one form of lymphomatosis.

1. Jungherr, E., Doyle, L. P., and Johnson, E. P., *Am. J. Vet. Res.*, 1941, v2, 116.
2. Furth, J., *J. Exp. Med.*, 1931, v53, 243.
3. ———, *Arch. Path.*, 1931, v12, 1.
4. Beard, J. W., Sharp, D. G., and Eckert, E. A., *Adv. in Virus Research*, 1955, v3, in press.
5. Eckert, E. A., Sharp, D. G., Beard, D., Green,

- I., and Beard, J. W., *J. Nat. Canc. Inst.*, in press.
6. Waters, N. F., *Poultry Science*, 1945, v24, 269.
7. Eckert, E. A., Beard, D., and Beard, J. W., *J. Nat. Canc. Inst.*, 1954, v14, 1055.
8. Mommaerts, E. B., Sharp, D. G., Eckert, E. A., Beard, D., and Beard, J. W., *ibid.*, 1954, v14, 1011.
9. Green, I., Beard, D., Eckert, E. A., and Beard, J. W., *PROC. SOC. EXP. BIOL. AND MED.*, 1954, v85, 406.
10. Eckert, E. A., Beard, D., and Beard, J. W., *J. Nat. Canc. Inst.*, 1955, v15, 1195.
11. Sharp, D. G., Eckert, E. A., Beard, D., and Beard, J. W., *J. Bact.*, 1952, v63, 151.
12. Sharp, D. G., and Beard, J. W., *Biochem. et Biophys. Acta*, 1954, v14, 12.
13. Sharp, D. G., Eckert, E. A., Burmester, B. R., and Beard, J. W., *PROC. SOC. EXP. BIOL. AND MED.*, 1952, v79, 204.

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Effects of Sodium Diphenylhydantoinate upon Isolated Small Intestine of the Rabbit. (21972)

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The effects of a number of drugs upon the rhythm of isolated rabbit intestine were studied. Among the drugs tested was sodium diphenyl-hydantoinate (Dilantin). This proved to have remarkable effects which, to our knowledge, have not been described previously.

Material and methods. The small intestine was rapidly removed from a freshly stunned rabbit. This was immediately placed into oxygenated Tyrode's solution. Lengths of duodenum were most generally employed, although jejunum and ileum were also used in some experiments. The unused portions were stored in cold Tyrode solution for later use. The length of the gut used was between 2 to 3 cm. One end of the gut was fixed by a silver hook which in turn was fastened to the walls of the chamber. The other end of the gut was led by a thread to a very sensitive myograph transducer. This in turn was connected to the amplifier of a Hoff-Geddes-Spencer Physiograph and the resultant recording was traced out by an ink-writing pen upon a moving strip of paper. The amplification factor for movement was about 200 at maximum gain. With the initial tension of the myograph spring overcome, a 200 mg weight produced a deflection of 7.5 mm at maximum gain.

The chamber enclosing the isolated gut had 3 openings at its base. The first of these

allowed for the continuous entry of oxygen into the solution. This kept the solution well-oxygenated and also served to mix the solution and any added drugs. An opening at the lowest part of the chamber allowed for drainage at will and a third opening provided for the entry of fresh Tyrode solution of pH about 7.6. A further portal in the upper part of the chamber enabled one to add drugs to the solution. The bath was kept at any constant temperature between 35° and 38°C by means of a heat lamp which was shone upon a blackened portion of the chamber. The intensity of this heating was regulated by means of a variac.

Results. The addition of Dilantin in amounts sufficient to produce a resultant concentration of 2 to 8 mg% (0.07 to 0.29 mmol/l) in the solution resulted in a rapid decrease in the amplitude of the contractions and a fall in tone (Fig. 1, A and B). This effect was noted within 15 to 30 seconds and the contractions generally disappeared within one minute. The addition of smaller amounts of Dilantin resulted in a more gradual decrease in the tone and in the amplitude of the contractions. A concentration of 0.5 mg % (0.018 mmol/l) was sufficient to reduce considerably the amplitude of contraction of the gut (Fig. 1, C). With the smaller concentrations of Dilantin, the gut contractions would become reduced in

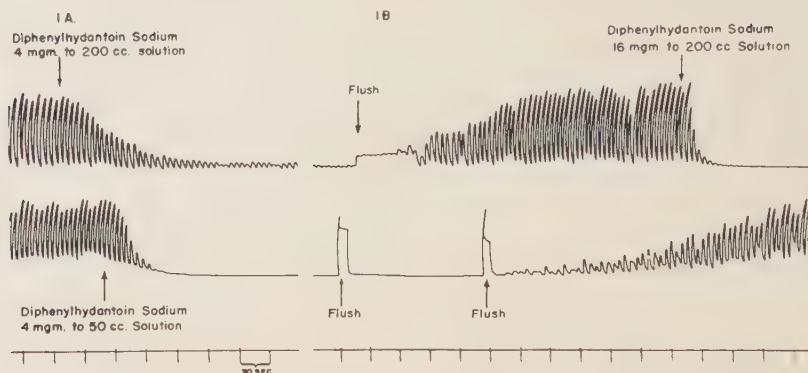


FIG. 1. A. Effects of Dilantin upon 2 separate samples of rabbit duodenum, both examined simultaneously. Note rapid decrease in amplitude of contractions and fall in tone upon addition of the drug; also note more rapid effects of higher concentrations. B. Note rapid return of contractions of normal amplitude upon flushing away the Dilantin. Gut exposed to higher concentration requires a longer time and more flushing before returning to normal level of contractions and of tone.

amplitude, but after several minutes the contractions would become fairly constant in strength.

Dilantin affects the rate as well as the amplitude of contractions. This effect is apparent in concentrations of the drug which markedly depress the amplitude of contractions without, of course, abolishing them (Fig. 2). By means of increasing the amplifying factor of the apparatus, it was possible to detect minute contractions of the gut. With concentrations of Dilantin of 6 to 8 mg%, the rate of contractions was reduced to one-third to two-thirds of the control rate.

The effects of Dilantin are rapidly revers-

ible. Even after the gut had been exposed to concentrations of the drug sufficient to prevent any recordable contractions over a period of over 50 minutes, removing the drug solution and replacing it with fresh Tyrode solution led to the appearance of small contractions within 75 seconds and a return to normal amplitude of contractions within 3 minutes. This reversibility of effect was present after repeated exposures to the Dilantin over a period of 8 hours.

To rule out the possibility that any of the effects noted were due to the alkalinity of the Dilantin, sodium hydroxide solution was added to the Tyrode solution bathing the gut in amounts sufficient to equal and, in some instances, exceed the pH of a solution of Dilantin concentrated enough to stop the contractions of the gut. Such an addition of base led to an increase in tone (Fig. 3A)(1). This increase in tone could be reversed and the tone returned to normal levels by the addition of Dilantin to the already alkaline solution (Fig. 3B). This would appear effectively to rule out simple alkalinity as the basis for the mechanism of action of the Dilantin.

The effects of the Dilantin were compared with the direct effects of sodium phenobarbital upon the isolated gut. In concentrations of 10 mg% (0.39 mmol/l), the sodium phenobarbital had a slight effect in reducing the rate of contraction from 16 per minute to 14

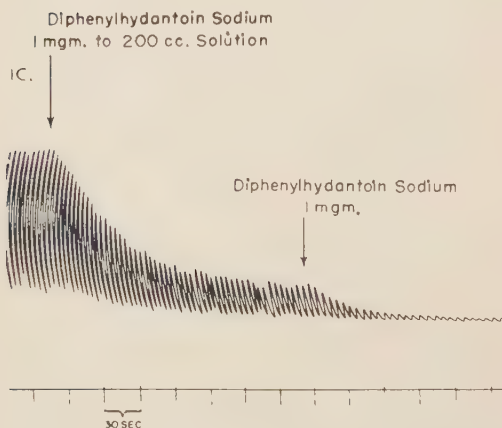


FIG. 1. C. Slower and lesser effect of smaller concentrations of Dilantin on amplitude and rate of contractions.

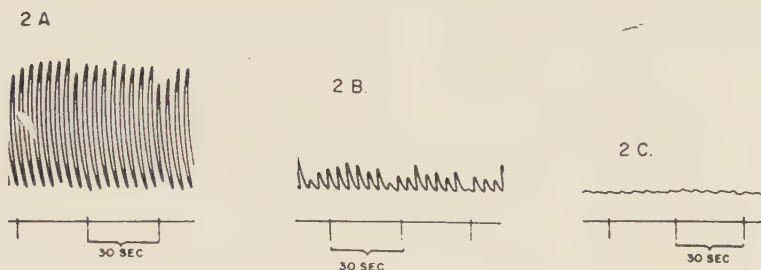


FIG. 2. A. Control record of contractions of gut. Note great amplitude of contraction and rate of 16 per min. B. In intermediate concentrations of Dilantin, amplitude of contraction is reduced. Rate is 15 per min. Amplification has been increased. C. In a concentration of 6 mg % Dilantin contractions of the gut are barely visible, even with the maximum gain of the amplifying system. The rate is 11 per min.

per minute, with some reduction in the amplitude of contraction as well. At a level of 21 mg% (0.83 mmol/l) there was a marked reduction in the amplitude of contractions and the rate was 14 per minute. The effects of the sodium phenobarbital upon the gut were much less rapidly reversible than those of Dilantin.

Discussion. A concentration of 0.5 mg%

of Dilantin was effective in reducing the amplitude of contraction of the isolated small intestine of the rabbit. Concentrations of 8 mg% abolished recordable contractions.

It has been reported(2) that the blood levels of hydantoinate in human patients receiving Dilantin for the treatment of epilepsy range between 3 to 6 mg%. Thus, it is obvious that the range of concentrations em-

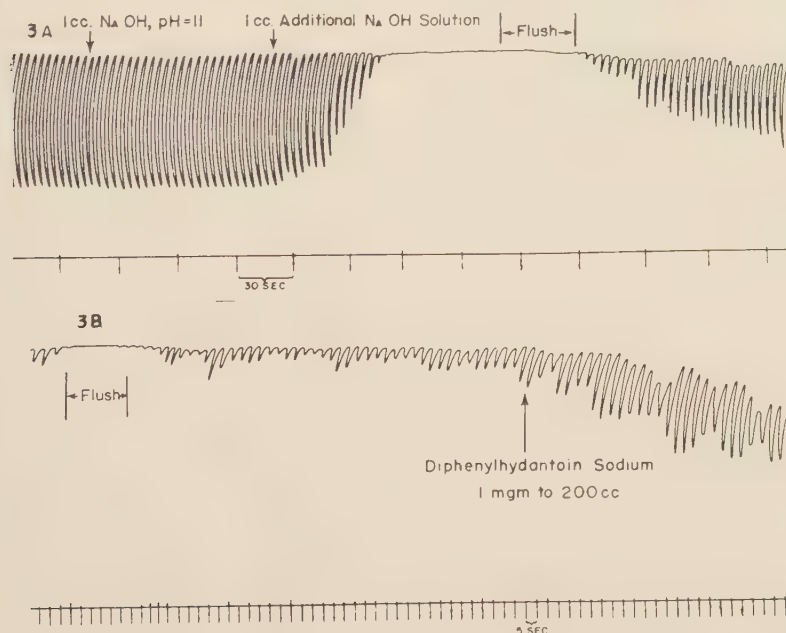


FIG. 3. A. Rise in tone upon addition of NaOH solution. Although the record suggests that contractions stopped with the increase in tone, this was not so. Gut continued contracting, but contractions were not recorded since normal tension range of this myograph was exceeded. This effect of NaOH is reversible by flushing. B. Flushing after addition of base leads to a slight reduction in tone. Addition of Dilantin leads to rapid fall in tone. Apparent increase in amplitude of contractions is artefactual and is due to tension exceeding recording range of the myograph in the earlier section of the record.

ployed in these experiments was well within therapeutic limits. This is of some practical significance, as these findings may help to explain the constipation which sometimes develops in patients taking Dilantin.

These results may also be of importance in relation to the problem of so-called "abdominal" epilepsy. Commonly accepted criteria for the diagnosis of this form of seizure include: (1) episodic attacks of abdominal pain, (2) "cerebral dysrhythmia" as revealed by electroencephalography, and (3) improvement with marked reduction or cessation of these attacks upon the institution of anti-epileptic medication, usually with Dilantin. The implication has been that improvement upon the institution of this medication is due to an effect upon the central nervous system. The possibility of such improvement being due to a direct effect of the medication upon the gut has not been given adequate consideration. If, as has been demonstrated in these experiments (*in vitro*), the Dilantin has a direct effect upon the gut (*in vivo*), then the third criterion is no longer necessarily valid in indicating the "central" origin of these attacks. The problem of "abdominal" epilepsy should be considered without regard for the

effects of anti-convulsant medication, unless the direct effects of this medication upon the gut can be dissociated from its effects upon the central nervous system.

These experiments may have clinical implications in other diseases affecting the gastro-intestinal tract, particularly those where increased motility or spasticity, or both, are prominent.

Summary. (1) Dilantin has a direct effect upon isolated rabbit gut. It reduces tone, reduces the amplitude and rate of contractions, and in higher concentrations can completely abolish the contractions. (2) The effect of Dilantin upon the isolated gut is rapidly reversible by simply replacing the gut in fresh Tyrode solution. (3) This effect of Dilantin is not due to its alkalinity. (4) Dilantin has a much more marked effect upon the gut than has sodium phenobarbital. (5) The clinical implications of these effects of Dilantin upon isolated gut are discussed briefly.

1. Gruber, C. M., *J. Pharm. Exp. Therap.*, 1926, v30, 149.

2. Kozelka, F. L., and Hine, C. H., *ibid.*, 1940, v69, 292.

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Propagation of Equine Abortion Virus in the Chick Embryo.* (21973)

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Doll(1) was the first to offer unequivocal proof that the virus of equine abortion (EAV) can be cultivated in the chorio-allantoic membrane of the chick embryo with intranuclear inclusions characteristic of the disease. The hamster-adapted variant used in his experiments would not induce infection in chick embryos by allantoic, amnionic or yolk-sac inoculation. Doll has pointed out that all attempts at propagation with infected equine fetal tissue have met with failure in the chick embryo. Previous experience(2) has shown

that a field strain of EAV can survive 8 serial passages in chick embryo lung and liver maintained in flasks. No inclusions could be identified in chick tissue but were demonstrated in fetal horse tissue inoculated with chick embryo passage material. In the present communication, the serial propagation of EAV in chick embryos is reported.

Material and methods. The virus used to initiate this study (strain F) had been adapted to the hamster liver(3). The liver inocula, hamster or chick embryo, were frozen and ground, diluted 1:10 in physiological saline and centrifuged lightly. Fertile eggs

* Aided by a Grant from the Grayson Foundation, Inc.

of 12-day incubation were injected with 10% liver suspension in the yolk and amniotic sacs with 0.5 ml and 0.1 ml respectively. Initially, the eggs were inoculated with hamster liver, and subsequently with chick embryo liver for 14 consecutive passages. Each serial passage was terminated 4-5 days following inoculation. In order to determine the pathogenicity of chick embryo material, alternate passages were inoculated into 3-week-old hamsters. Representative pieces of liver from the hamster and chick embryo passages were fixed in Zenker-acetic acid for histological studies.

Complement-fixation methods. The original complement-fixation procedure(4) for equine abortion employing the 50% hemolysin end-point method, was abandoned because of the excessive amount of reagents required. Doll later employed the conventional method(5). The latter procedure was used in the present study to save materials. The liver antigens were ground in physiological saline and stored at -50°C . Prior to use they were centrifuged at 2500 rpm for 10'. The origin and preparation of the positive and negative sera, and control antigens, have been previously documented(3,6). Essentially, the procedure utilized 2-fold 0.25 ml serial dilutions of antigen, 0.25 ml of 1:10 dilutions of antiserum of equine origin and 2 full units of complement in 0.5 ml. Fixation was carried out overnight at 4°C and 0.5 ml of sensitized cells were added for 1 hour at 37°C . Adequate controls were used for each reagent, including normal horse serum.

Results. Serial propagation in chick embryos. No significant mortality was observed in any of the trials. The search for intranuclear inclusions characteristic of the infection in susceptible species was unrewarding in early experiments. In the 6th and subsequent passages intranuclear inclusions were identified in liver parenchymal cells. They were indistinguishable from those seen in the horse(6). Inclusions were occasionally numerous, usually focal in distribution and not abundant.

Identification of embryo propagated virus. Hamster adapted equine abortion virus is quite pathogenic for young hamsters with LD_{50} titres varying between 10^{-6} and 10^{-8} .

TABLE I. Complement Fixation Reactions.

Antigens	Antigen titration end-points
Equine fetal lung (abortion virus)	128
Normal fetal lung	4
14th passage chick embryo liver	Anticomplementary
Normal chick embryo liver	"
Hamster liver inoculated with 14th passage chick embryo	64
Normal hamster liver	8

Intranuclear inclusions are constant and abundant in the parenchymal cells of the liver. Chick embryo passage material remained pathogenic for 3-week-old hamsters, but the results were variable. Ten per cent chick embryo liver rarely killed all inoculated animals. The liver from all dead animals contained abundant typical inclusions. Doll(7) reports that chick embryo EAV loses pathogenicity for weaned hamsters. The dilution of the original hamster material during serial passage in chick embryo exceeded 10^{-15} . The dilution is only approximate since the routes of inoculation preclude accurate calculation. The known limiting dilution of infectivity was greatly exceeded, showing that the agent has been propagated in the chick embryo. The chick embryo adapted virus was further identified by the complement-fixation reaction. The data shown in the table present proof that the specificity of the egg adapted virus was maintained. The antigen titration end-point is defined as the highest dilution of antigen which resulted in complete fixation of complement with a 1:10 dilution of antiserum of equine origin.

Summary. The adaptation of hamster modified equine abortion virus to the chick embryo is reported. Characteristic intranuclear inclusions were identified in the liver parenchymal cells of chick embryos and hamsters receiving serial passage chick embryo liver. Identification of the virus is substantiated by the use of the complement-fixation reaction.

1. Doll, E. R., and Wallace, M. E., *Cornell Vet.*, 1954, v44, 453.
2. Randall, C. C., unpublished data.
3. Doll, E. R., Richards, M. S., and Wallace,

M. E., *Cornell Vet.*, 1953, v43, 551:

4. Randall, C. C., McVickar, D. L., and Doll, E. R., *PROC. SOC. EXP. BIOL. AND MED.*, 1950, v75, 465.

5. Doll, E. R., McCollum, W. H., Wallace, M. E., Bryans, J. T., and Richards, M. S., *Am. J. Vet. Res.*,

1953, v14, 40.

6. Randall, C. C., Ryden, F. W., Doll, E. R., and Schell, F. S., *Am. J. Path.*, 1953, v29, 139.

7. Doll, E. R., personal communication.

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Determination of Specific Activity of Sodium in Bone.* (21974)

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Studies of the role of skeletal sodium have been hampered by technical difficulties encountered in the analysis of this tissue for sodium content. These difficulties arise largely from the fact that bone contains tremendous amounts of calcium, the molar Ca/Na ratio being of the order of 25/1. This quantity of calcium causes serious interference with both the flame photometric and gravimetric methods for determination of sodium; and phosphate, in the quantities present in bone, may also interfere. Radioactivity measurements require large corrections for self-absorption if unaltered solutions of bone ash are used, and if attempts are made to precipitate calcium and phosphorus, as much as 20% of the radio-sodium present may be removed by co-precipitation(1).

In an attempt to circumvent the difficulties inherent in analysis of bone for sodium, a new method was developed in which interfering substances are removed by cation exchange chromatography(2). Under the conditions employed, sodium appears in the eluate entirely free from phosphate, calcium, magnesium and potassium, whereupon the sodium-containing fraction can be easily analyzed by flame photometry.

The present report extends this investigation to include the behavior of radioactive sodium on the column, and further defines the accuracy and limitations of the method.

Method. Column characteristics — A column 1.3 x 60 cm is employed, containing 27 g (oven-dried basis) of the sulfonated polysty-

rene resin Dowex 50-x12 (50-100 mesh). The resin rests on a plug of Pyrex glass wool and fills the column to a height of 42 cm. Details of the preparation and operation of the column and the collection of eluate fractions are given in a previous publication(2). **Analysis of Effluent Fractions.** Analyses for Na²³ and for K and Mg were carried out by standard methods (for references see 2). Calcium was determined by the hexanitratocerate technic (3). For purposes of this study, a special hood was fitted to the Weichelsbaum-Varney flame photometer in order to exhaust radioactive fumes to the outside. Measurements of Na^{22†} were made by pipetting 2 ml samples of eluate into 1.5 x 4.5 cm vials, which were then counted directly in a well-type scintillation counter. In instances where the activity was low, the sample was first evaporated on a steam bath to the appropriate degree of concentration. A known aliquot of Na²², appropriately diluted in NaCl solution, served as a standard. Decay corrections were obviated by counting the eluate fractions and the standard solution on the same day. The usual corrections for coincidence effects were applied. **Preparation of Bone Samples.** Bone is first dried and then ashed overnight at 525°C in a muffle furnace. The ash is dissolved in 3 N HCl, and the resulting solution boiled for 10 minutes. This solution is then placed on the column, any remaining dark particles having been removed by filtration through Pyrex glass wool, allowed to flow into the resin bed, and elution with 0.8 N HCl be-

* Supported by a grant from the Playtex Park Research Foundation

† Radiosodium²² was obtained from the Oak Ridge National Laboratories.

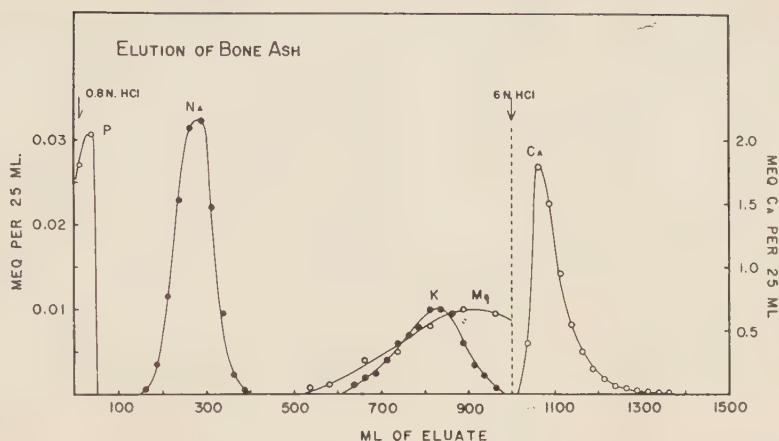


FIG. 1. Elution pattern of beef bone ash solution (0.5 g wet bone). Eluate collected in 25 ml fractions. Scale on the left-hand ordinate reads directly for Na and Mg, and should be multiplied by 0.1 for K. Phosphorus is plotted in $\text{mg} \times 10^{-3}$.

gun. Pyrex-brand glassware is used throughout.

Results. Elution of Bone Ash Solutions. A representative elution curve for a solution of bone ash (representing 0.5 g of wet beef bone)[†] is illustrated in Fig. 1. Phosphate is found only in the 0-50 ml range and K in the 600-1000 ml range. Magnesium first appears at the 525 ml. mark and is not completely eluted by 1000 ml. Calcium does not appear by the 1000 ml mark, and requires 400 ml of 6 N HCl to effect a complete recovery. The

100-400 ml fraction which contains the sodium is thus free from P, K, Mg and Ca. No attempt was made to test for the presence of other ions.

Fig. 2 illustrates the sodium-containing fraction of the eluate from a solution of bone ash to which a known amount of Na^{22} had been added. Both isotopes follow the same elution pattern with no appreciable change in the $\text{Na}^{22}/\text{Na}^{23}$ ratio as the elution proceeds.

In Fig. 3 a series of elution curves for Na^{22} (approximately $0.4 \mu\text{c}$ in each instance) added to bone samples of varying size are depicted. The position of the elution curve remains fairly constant from one run to the next. However, it was found that the Mg curve shifted markedly with variation in sample size. Its position for 0.5 g of bone is indicated in Fig. 1. When 0.75 g of bone were used, Mg first appeared in the 400-425 ml fraction and with 1 g, it was present by the 300 ml mark. In a previous study(2), it was noted that as the sample size was increased, calcium appeared much earlier.

Recovery of Added Na^{22} . Recoveries of radiosodium added to solutions of bone ash and to a known quantity of NaCl are listed in Table I, the data indicating a high degree of accuracy for the procedure as we have employed it. In the instance in which NaCl was used, it was found that the specific activity of the sodium added to the column corresponded very closely to that recovered, the two values

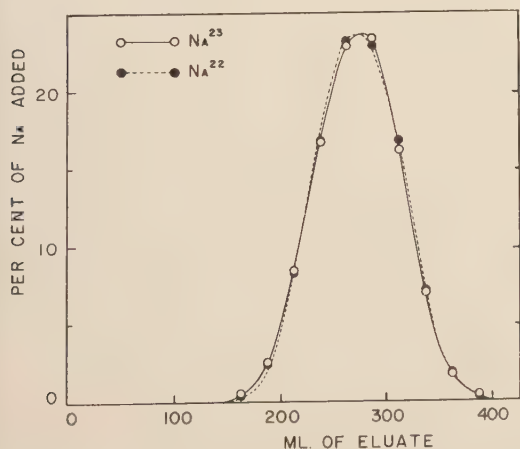


FIG. 2. Sodium elution pattern of beef bone ash solution (0.5 g wet bone) to which Na^{22} had been added. Eluate collected in 25 ml fractions. Elutriant 0.8 N HCl.

[†] The bone powder used was found by analysis to contain 12.2 meq. Ca and 0.25 meq. Na/g wet weight.

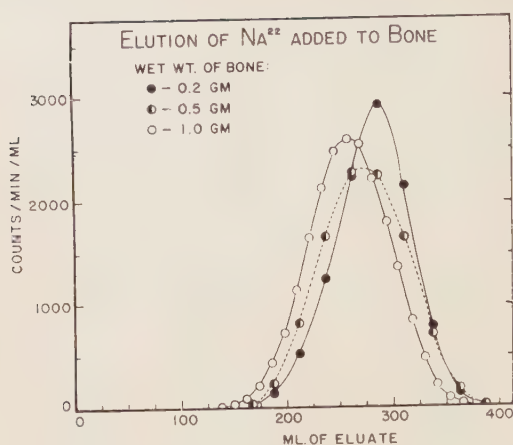


FIG. 3. Effect of sample size on sodium elution pattern. Elutrient 0.8 N HCl. Eluate collected in 12 ml or 25 ml fractions.

being 9.0×10^5 and 8.8×10^5 cts/min/meq. Na respectively.

Procedure adopted for determination of specific activity of bone samples. Bone is prepared as indicated above. The solution of bone ash is added to the column in a volume not to exceed 10 ml and allowed to flow into the resin bed. Elution with 0.8 N HCl is then carried out at a flow rate of 2.0 ± 0.5 ml per minute. The 0-100 ml eluate fraction is discarded. The 100-400 ml fraction is collected quantitatively and used for radioactivity measurements and for Na analysis by flame photometry. The elutrient is then changed to 6 N HCl in order to elute other cations more rapidly, and at least 400 ml is run through in order to completely elute K, Mg and Ca.

TABLE I. Recovery of Radosodium.

Sample	Na ²² added, cts/min.	Na ²² re- covered, cts/min.	% recovery
	$\times 1000$		
NaCl, 0.3 mM	270.7	268.8	99.4
Bone, 1 g	263.9	261.6	99.2
1	289.9	291.9	100.7
1	288.1	293.2	101.8
1	255.0	254.9	99.9
1	251.9	253.9	100.7
.5	251.9	256.7	102.0
.2	252.8	256.3	101.4
.75	252.8	255.0	100.9
.5	244.5	246.6	100.8
	Avg		100.7%
	Stand. dev.		.94
	" error		.30

Approximately 300 ml of 0.8 N HCl are then run through the column in order to re-equilibrate the resin with the weaker acid. The resin is then agitated by repeated inversions of the column, whereupon it is ready for the next run. The entire procedure, including the reconditioning of the resin, occupies about 7 hours, during which time the column requires very little attention.

The Na-containing fraction of eluate is thoroughly mixed, and an aliquot evaporated to dryness on a steam bath in order to drive off most of the HCl. The residue is made up to volume with water and analyzed for Na²³ by flame photometry. Depending on the amount of radioisotope present, radioactivity measurements are then made on another aliquot either in the original state, or after suitable concentration or dilution.

Discussion. It is evident that Na²² and Na²³ behave in similar fashion on this cation exchange column, and that measurements of the specific activity of sodium in bone are feasible by this method. Interfering substances relative to Na²³ analysis are removed by the procedure as we have employed it, and the necessity for large self-absorption corrections in Na²² analysis has been obviated since the Na-containing fraction of the eluate has a low solute content.

There are certain limits to sample size for a column of these dimensions. Analyses for Na²³ gave distinctly less accurate results when amounts of the order of less than 0.06 meq. were added to the column(2), presumably because of the small but definite reagent blank. According to the results of our previous study on the composition of rat bone, sample size should therefore be not less than 0.5 g (wet weight) for very young animals and 0.25 g for adults(2). The upper limit depends on whether a slight degree of Mg contamination of the Na fraction can be tolerated; if not, the sample size should not exceed 1.5 g for young animals and 0.75 g for adults. The marked shift in the calcium curve places an absolute upper limit at 4 and 2 g, respectively, for the 2 age groups.

Since different batches of Dowex 50 may differ somewhat, elution curves should be run prior to using this method for analysis of bone

for sodium. In this connection it should be noted that the elution characteristics of the column we are now using differ slightly from those depicted previously(2).

Conclusion. A new method for the determination of specific activity of sodium in bone is presented. The method consists of placing a solution of bone ash on a cation exchange column. Elution with acid results in the quantitative recovery of both stable and radioactive sodium in a certain fraction of the eluate; this fraction is free from P, K, Mg and Ca and can be easily analyzed for both sodium and radiosodium.

The scintillation counter equipment was made available to us through the kindness of Mr. J. Russell Hayes of the Medical Division, Atomic Energy Project of the University of Rochester, and the specially adapted flame photometer by Dr. Taft Toribara of the Radiation Biology Division.

1. Davies, R. E., Kornberg, H. L., and Wilson, G. M., *Biochem. J.*, 1952, v52, xv.

2. Forbes, G. B., and D'Ambruso, Margaret, *J. Biol. Chem.*, 1955, v212, 655-661.

3. Kochakian, C. D., and Fox, R. Phyllis, *Indust. and Eng. Chem., Anal. Ed.*, 1944, v16, 762.

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Oestrogenic Activity of 16 Epi-Oestriol.*† (21975)

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The isolation of 16 epi-oestriol (oestra-1:3:5-triene-3:16B:17B-triol) from the acid hydrolyzed urine of pregnant women was described by Marrian and Bauld(1). Since the yield of this new Kober Chromagen was 0.1 mg/l and since this substance seems identical with 16 epi-oestriol as prepared by Huffman(2) considerable impetus was given to the present investigation in an effort to ascertain its activity on the growth potential of the uterus of the ovariectomized rat. Vaginal smears were also studied quite extensively. Moreover, since practically no biological information exists in the scientific literature on this naturally occurring oestrogen it seemed of particular significance at this time to elaborate its biological activity.

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§ The technical assistance of Miss Nancy M. Raney and Miss M. Claire Simoneau is gratefully acknowledged.

Procedures. Virgin, albino rats, approximately 100 days of age, were used in these experiments. They were started on treatment 7 days after bilateral ovariectomy. The 16 epi-oestriol was dissolved in U.S.P. propylene glycol, and injected subcutaneously once daily for 3 days. Vaginal smears were taken daily, and 72 hours after the first injection necropsies were performed. The uteri were removed quickly and slit longitudinally on bibulous paper, thus removing excess uterine luminal fluid. The uteri were then weighed to the nearest 0.2 mg on a Roller-Smith torsion balance. After the wet weights were determined, the uteri were placed in an oven at 110°C for dry weight determinations. A standard period of 24 hours at 110°C was used uniformly for drying each uterus. After this period the weight of each uterus was determined on a Roller-Smith torsion balance.

Results. A. Effect of 16 epi-oestriol on vagina of the ovariectomized rat. These results clearly indicate that 16 epi-oestriol has the ability to induce oestrogenic changes in the vagina of the ovariectomized rat (Table I). A demonstrable effect can be observed with 1.0 µg of this substance. Additional

TABLE I. Influence of 16 Epi-oestriol on Vagina of the Ovariectomized Rat.

Daily dose of 16 epi-oestriol, μg	Vaginal smear			
	24 hr after 1st inj.	48 hr after 1st inj.	56 hr after 1st inj.	72 hr after 1st inj.
.00 (1 cc propylene glycol)	L+++E	L++++	L++++	L++++
.01	L++C+	L++E+C+	L++E+C+	L++E+C+
.1	L++C+	L++C+	L++C+	L++C+
1	L++E+C+	L++E+C+	L++E+C+	L++E+C+
5	L+C++	L+E+C+	L+E++C+	L+E+C++
10	L+EC++	LE++C+	L+EC++	LE++C++
25	L+E+C++	"	"	LE++C++
30	"	L+C++	L+C++	L+C++
40	L+C++	LC++	LC++	C++++
50	L+EC++	LE++C+	"	LC++++
100	L+E+C++	"	"	"
.1 oestradiol 17 β	L++E+	L+E+C++	L+EC++	C++++

L = Leucocytes; E = Nucleated epithelial cells; C = Cornified cells.

The pluses indicate the ratio of cell types found in the vaginal smear. For example: L+E+C++ = considerably more cornified cells than leucocytes and nucleated epithelial cells.

doses of 16 epi-oestriol produced a more definitive oestrous pattern, whereas 3 daily doses of 40 μg effected a full-blown estrous pattern in the vagina. Comparatively, this material is a very weak oestrogen, especially when one compares it with the vaginal cornifying effects of the various oestrogens(3,4). Of particular significance is the fact that 72 hours were required for the induction of a full vaginal oestrous smear (Table I).

B. Influence of 16 epi-oestriol on uterus of the ovariectomized rat. That this substance is a very weak oestrogen is further borne out by the information obtained from the wet and dry uterine weights. A slight, but statistically significant, increase in both the wet and dry uterine weights was observed in those animals receiving 1.0 μg of this material (Table II). A further increase in uterine weight was observed in the group of rats given 10 μg , and the maximum response was observed when 40 μg were administered daily for 3 days. Interestingly enough, parallel results were obtained with the various dosages of 16 epi-oestriol for both the vaginae and uteri (Tables I and II).

Included in Table II is the effect of injections of oestradiol 17 β on the uterus of the ovariectomized rat. A dramatic uterine response, far above that obtained with 400 times (on a weight relationship basis) the amount of 16 epi-oestriol, was achieved with oestradiol.

The increase was quite marked regardless of the vehicle used, sesame oil or propylene glycol (Table II).

Discussion. These results show quite conclusively that 16 epi-oestriol is the weakest of all known naturally occurring oestrogens described thus far(3-5). The exact function of this oestrogen is not known at this time, but it would not be too conjectural to think of this substance as a possible link in the metabolism of the oestrogens. Furthermore, while the recent results of Hisaw, Velardo, and Goolsby (5) indicated that oestriol had the ability to markedly restrict the action of oestradiol 17 β on the growth of the uterus of the ovariectomized rat, preliminary results at hand seem to suggest quite strongly that this compound (16 epi-oestriol) shares in common with oestriol the property of restricting the uterine growth promoting action of oestradiol 17 β . Additional research on this phase of our work is now in progress.

Summary. The oestrogenic activity of 16 epi-oestriol was determined. Virgin, adult albino rats were ovariectomized, and 7 days later were put on experiments. A dose-response (0.01 to 100 μg) of this substance was determined utilizing the uterus and vagina as end points. The results from these experiments indicate that 1.0 μg of this oestrogen produces a slight effect on the vagina as well

TABLE II. Influence of 16 Epi-oestriol on Uterine Growth in Ovariectomized Rats.

Treatment	No. of animals	Uterine wet wt (mg)	Uterine dry wt (mg)
Control, 10 full days after ovariectomy	25	115.5 \pm 3.3*	22.4 \pm .65
.1 μ g oestradiol 17 β in Sesame oil	36	227.3 \pm 6.1	42.3 \pm 1.8
.1 " in prop. glycol	15	236.0 \pm 1.0	44.4 \pm 2.5
.01 16 epi-oestriol	8	143.0 \pm 7.2	27.0 \pm 2.1
.1	15	126.4 \pm 5.2	27.2 \pm 1.5
1	8	150.0 \pm 8.6	26.1 \pm .8
5	8	142.7 \pm 6.8	25.8 \pm 1.7
10	9	171.4 \pm 8.7	28.5 \pm 1.6
25	9	164.3 \pm 5.1	30.5 \pm 1.1
30	7	167.2 \pm 8.3	32.8 \pm 2.0
40	7	181.6 \pm 8.0	33.0 \pm 2.0
50	7	175.1 \pm 8.1	32.6 \pm 1.9
100	6	180.2 \pm 7.3	30.6 \pm 1.5

* Avg \pm stand. error.

as on the uterus. Three daily doses of 40.0 μ g of 16 epi-oestriol produced a maximal effect on the vagina (full cornification) and the uterine weight. This substance seems to be the weakest of all the known naturally-occurring oestrogens described thus far.

1. Marrian, G. F., and Bauld, W. S., *Biochem. J.*, 1955, v59, 136.

2. Huffman, M. N., *J. Amer. Chem. Soc.*, 1942, v64, 2235.

3. Sondern, C. W., and Sealey, J. L., *Endocrinology*, 1940, v27, 670.

4. Sealey, J. L., and Sondern, C. W., *ibid.*, 1941, v29, 356.

5. Hisaw, F. L., Velardo, J. T., and Goolsby, C. M., *J. Clin. Endo. and Metab.*, 1954, v14, 1134.

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Pentose Dependence of *Lactobacillus gayonii* and Related Species for Early Growth.* (1976)

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Requirements for early growth of *Lactobacillus gayonii* and the closely related species, *buchneri*, *lycopersici*, *fermenti*, *mannitopoeus* and *brevis* have been investigated recently in the authors' laboratory because preliminary screening experiments showed this group of organisms to be particularly well suited for studies of D- α -hydroxy fatty acid and related lipid metabolism. The latter investigations, which were prompted by the finding that D- α -hydroxy fatty acids are essential in the nutrition of *Lactobacillus casei* 280-16(1,2),

are to be described in subsequent reports. The effects of pentoses were studied because it had been shown previously that these sugars promote better growth and acid production than does glucose for this particular group of microorganisms(3), and it has now been found that although glucose may be utilized adaptively, an appropriate pentose is essential for early growth of most of these species.

Methods. The sugars tested were D-glucose (C.P. dextrose, J. T. Baker Chemical Co.), L-arabinose (Pfanstiehl Chemical Co.), D-arabinose, L-xylose, D-xylose, and D-ribose (products of Nutritional Biochemicals Corp.). Sugar solutions of appropriate concentration were added to 13 x 100 mm soft glass test tubes together with sufficient water to make 1.0 ml

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per tube and sterilized in the autoclave. The test medium, consisting of enzymatically digested casein (N-Z-Case, Sheffield Farms Co.) 0.6%, yeast extract (Difco) 0.2%, ammonia-hydrolysed yeast nucleic acid(4) 0.02%, glucose 0.5%, vitamin-mineral mixture(5) 20.6 mg %, and buffer solution(5) 5.0 ml %, was made to two-thirds final volume, autoclaved 15 minutes at 15 lb and added (2 ml per tube) aseptically to the previously sterilized tubes. The test microorganisms, described previously (6),[†] were maintained for the 10 years preceding the present experiments by serial subculture at 3-week intervals in Bacto-tomato juice agar (Difco) (the fresh stabs were incubated 40 hours then stored in a refrigerator). An inoculum medium was prepared by making a uniform suspension of dehydrated Bacto-tomato juice agar in the amount of water (cold) recommended by the manufacturer, stirring it for 10 minutes and then filtering to remove the agar, which, under these conditions, remained mostly undissolved. Fifteen ml aliquots of the filtrate were distributed into 50-ml centrifuge tubes and sterilized. Eighteen- to 24-hour cultures of the test organisms in this medium were centrifuged and resuspended in sufficient sterile saline to yield approximately 10^8 cells per ml. One-tenth ml of suspension per tube was employed as the inoculum. The inoculated tubes were incubated in a precision constant temperature water bath at 35°C.

Results. Bacto-tomato juice agar was employed as the inoculum medium (agar removed) to avoid adaptations during growth of the inoculum which might not have already occurred during maintenance of the test microorganisms. It is of interest in this respect that the composition of Bacto-tomato juice agar (tomato juice solids 2%, Bacto-peptone 1%, Bacto-peptonized milk 1%, agar 1.1%) includes tomato juice as the sole source of utilizable carbohydrate for most of these organisms since lactose, presumably present in peptonized milk, is not utilized appreciably by this group with the exception of *L. fermenti*(3).

No report has been found concerning the types of sugar present in tomatoes, but it seems likely that pentoses are present in Bacto-tomato juice agar since the test organisms, as will be shown further on, remain unadapted to glucose utilization. The total sugars present in tomatoes average about 3.4% according to Chatfield and Adams(7).

Yeast extract was included in the test medium since it has been shown previously to stimulate growth of lactic acid bacteria(3), and ammonia-hydrolysed yeast nucleic acid was employed as a nucleotide supplement, since the latter is known to stimulate growth of *L. gayonii*(4). The growth rates of *L. brevis*, *L. buchneri*, *L. gayonii* and *L. mannito-poeus* were determined in the test medium with L-arabinose as the sugar source with and without the yeast extract and ammonia-hydrolysed yeast nucleic acid, and the stimulatory effects of these supplements (data not shown) were found to be independent and additive except with *L. buchneri*, for which the ammonia-hydrolysed yeast nucleic acid appeared to be inert. The complete test medium (containing both supplements) was used in subsequent experiments unless otherwise specified.

Glucose (0.5%) was autoclaved with the medium in all of the tests (unless otherwise noted) since Rogers *et al.*(8) have shown that an unidentified substance essential for early growth of *L. gayonii* is formed in glucose containing media upon heating. The concentrations of the various sugars tested (including glucose) were in addition to this basal glucose level, but the test sugars were sterilized separately from the medium to avoid alteration through "browning".

The data shown in Fig. 1 illustrate that *L. brevis* (B), *L. gayonii* (G), and *L. mannito-poeus* (M) respond markedly to L-arabinose (a) in 16 hours, but negligibly to glucose (g) under these conditions. Similar effects were noted with *L. buchneri* and *L. lycopersici* (data not shown), but *L. fermenti* exhibited a considerable 16-hour response to glucose, exceeded only by that to D-xylose and D-ribose.

All test organisms responded ultimately to glucose, although *L. buchneri* was slowest in doing so. The data presented in Fig. 2 show

[†] The American Type Culture Collection No. of *L. brevis* is incorrectly given as 8257 in the reference cited. The correct number is 8287.

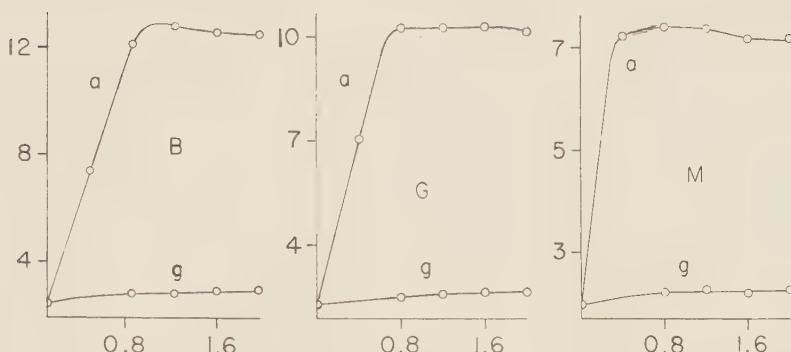


FIG. 1. 16 hr response of *L. brevis* (B), *L. gayonii* (G) and *L. manniopoeus* (M) to L-arabinose (a) and D-glucose (g). Values on horizontal scales are sugar concentrations (%); those on vertical scales are titrations (calculated as ml of 0.01 N NaOH required to neutralize 1 ml of culture).

that the response of *L. buchneri* to glucose (g) was still only slight after 145 hours, whereas D-xylose (x) was nearly completely fermented in 145 hours and L-arabinose (a) in 50 hours.

Eighteen-hour responses of each of the test organisms to 1% L-arabinose, D-ribose, D-xylose and D-glucose, respectively, are given in Table I (the responses to D-arabinose and L-xylose were excluded from the table because they were negligible in all cases). L-Arabinose yielded the best or nearly the best response for all the test organisms except *L. fermenti*, and D-ribose and D-xylose were generally much better utilized than D-glucose (although some utilization of the latter was already in evidence).

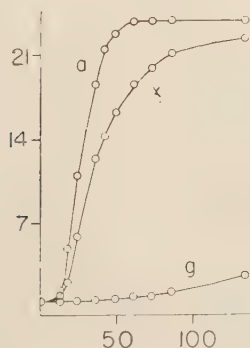


FIG. 2. Growth of *L. buchneri* with 2% L-arabinose (a), 2% D-xylose (x), and 2% glucose (g). Values on horizontal scale are hours of incubation; those on vertical scale are titrations (calculated as ml of 0.01 N NaOH required to neutralize 1 ml of culture). The yeast extract, the ammonia-hydrolysed yeast nucleic acid and the initial supplement of glucose were omitted from the test medium in this experiment.

Results in the test medium solidified with agar and supplemented with D-glucose and L-arabinose, respectively, showed the same trends as in liquid medium. Colonies of *L. brevis*, *L. gayonii* and *L. manniopoeus* in L-arabinose-supplemented agar medium were large (approximately 3 mm diameter) after 40 hours incubation under carbon dioxide, whereas those in D-glucose-supplemented agar medium were still nearly microscopic after this length of time. Colonies in the latter medium ultimately reached normal size, however, and the final number of colonies per unit volume of inoculum suspension were approximately the same with either D-glucose or L-arabinose, indicating that probably each cell in the inoculum suspension possessed an adaptive mechanism for the utilization of D-glucose.

The relatively large amounts of pentose required to promote growth of the test organisms (Fig. 1) suggest that the pentose functions as an energy source, rather than as a vitamin-like nutrient. A study of the enzyme systems involved has not been attempted, but a reasonable hypothesis appears to be that these pentose-requiring organisms possess a constitutive pentokinase together with an adaptive hexokinase. It seems unlikely that the growth-promoting effects of the pentoses were due to contamination with vitamin-like growth factors since the test medium contained yeast extract, which is generally found to be an adequate source of such unidentified nutrients. This possibility was rendered still less likely by the use of recrystallized L-arabinose, which

TABLE I. 18-Hour Response of the Test Organisms to 4 Sugars (at 1% Concentration).

	Growth response (acid production)*			
	L-Arabinose	D-Ribose	D-Xylose	D-Glucose
<i>L. brevis</i>	14.25	14.13	12.42	2.80
<i>L. buchneri</i>	10.28	4.87	4.80	.67
<i>L. fermenti</i>	2.38	9.81	8.17	5.33
<i>L. gayonii</i>	11.28	11.92	6.42	2.60
<i>L. lycopersici</i>	3.40	1.30	2.53	2.10
<i>L. mannitopoeus</i>	13.48	8.42	10.13	2.79

* Calculated as ml of 0.01 N NaOH required to neutralize 1 ml of culture.

was found to have retained all of its original growth-promoting activity.

That D-glucose has been employed with general success as a carbohydrate source for these and related microorganisms may be accounted for by adaption to D-glucose resulting under the test conditions employed. Cheldelin and Riggs(9), for example, carried their culture of *L. gayonii* (A.T.C.C. 8289) in a medium containing 1% glucose, 1% yeast extract, and 2% agar and, like most other researchers, employed an inoculum medium also containing glucose as the sole carbohydrate source. Such conditions would, of course, be certain to maintain this organism in its maximally adapted state.

Summary. Evidence has been presented indicating that an appropriate pentose is essential as an energy source for early growth of *L. brevis*, *L. buchneri*, *L. gayonii*, *L. lycopersici* and *L. mannitopoeus* if passage through a

pentose-free glucose-containing medium, which would promote adaptive utilization of glucose, is avoided.

1. Camien, M. N., and Dunn, M. S., *J. Biol. Chem.*, 1953, v201, 621.
2. ———, *ibid.*, 1954, v211, 593.
3. Camien, M. N., Dunn, M. S., and Salle, A. J., *ibid.*, 1947, v168, 33.
4. Hutchings, B. L., Sloane, N. H., and Boggiano, E., *ibid.*, 1946, v162, 737.
5. Camien, M. N., and Dunn, M. S., *Proc. Soc. Exp. Biol. and Med.*, 1954, v85, 177.
6. Dunn, M. S., Shankman, S., Camien, M. N., and Block, H., *J. Biol. Chem.*, 1947, v168, 1.
7. Chatfield, C., and Adams, G., *U. S. Dept. Agr.*, 1931, *Circ. No.* 146.
8. Rogers, D., King, T. E., and Cheldelin, V. H., *Proc. Soc. Exp. Biol. and Med.*, 1953, v82, 140.
9. Cheldelin, V. H., and Riggs, T. R., *Arch. Biochem.*, 1946, v10, 19.

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Effectiveness of Phenoxymethyl Penicillin V, and Sodium Penicillin G Against Hemolytic Streptococcus Infection in White Mice. (21977)

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The following experiments were conducted to determine the comparative effectiveness on a weight basis of phenoxymethyl penicillin V, and sodium penicillin G against hemolytic streptococcus infection in white mice.

Materials and methods. Two groups totaling 640 white mice in 2 tests were injected intraperitoneally with a 10^{-5} dilution of a 16-hour blood broth culture of hemolytic streptococcus C-203. This is usually some-

what more than 1000 LD₅₀, but less than 10,000 LD₅₀. In one test indicated below it was actually 4,050 LD₅₀, and in the other test it was 5,900 LD₅₀ according to virulence tests which utilized 100 mice each. Following injection of the virulent culture, subgroups of 20 mice each were injected immediately by the subcutaneous route with either one or the other of the above penicillins. Different subgroups of 20 mice received either 7 or 9 2-fold

TABLE I. CD_{50} * of Two Penicillins in Hemolytic Streptococcus Infected Mice.

Penicillin used	1 dose therapy (mg)	2 dose therapy (mg)
Phenoxymethyl penicillin V	.207	.031
Penicillin G	.183	.030

CD_{50} computed by the method of Reed and Muench(1).

differing penicillin dosages in the range of 0.5 mg to 0.00095 mg. Stock solutions of the penicillins were furnished and prepared just before use in the mice by Dr. M. D. Bray of these laboratories. In one comparative test of the 2 penicillins, the mice got a single dose of penicillin, given at the time of infection. In the second test, a second dose of penicillin was given 5 hours after the first dose.

Results. As shown in Table I, a CD_{50} (dose curing 50% of mice) is indicated for

the 2 penicillins from the results of these two tests. There was no significant difference in the therapeutic efficacy of phenoxymethyl penicillin V and penicillin G in the subcutaneous treatment of hemolytic streptococcus infections in white mice. The CD_{50} doses of the two were comparable, whether on single or double injection 5 hours apart. Furthermore the death rate in mice injected with subcurative doses of these two penicillins did not differ significantly.

Conclusion. Phenoxymethyl penicillin V and penicillin G have essentially the same curative properties against hemolytic streptococcus infections in white mice when these antibiotics are injected subcutaneously.

1. Reed, L. J., and Muench, H., *Am. J. Hyg.*, 1938, v27, 493.

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Hemolysin Production in Irradiated Mice Given Spleen or Bone-Marrow Homogenate. (21978)

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Antibody formation is inhibited by exposure of the whole body to ionizing radiation (1,2), and the degree of inhibition is correlated with the time of antigen administration referred to the time of radiation exposure (3,4). Spleen or appendix shielding in rabbits during exposure to midlethal amounts of X-radiation resulted in a marked retention of the capacity to produce antibody to sheep erythrocytes(5). Preparatory treatment of the antigen with tissues or extracts of cells has recently been shown to improve the immune response in irradiated rabbits(6,7). Treatment of radiation injury in mice with spleen shielding(8) and spleen or bone marrow homogenates(9,10) accelerates hematopoietic recovery, lowers radiation mortality and has been shown to aid in the defenses against experimental infection(11). The present work was undertaken to study the hemoly-

sin response in irradiated mice and to see whether the injection of marrow or spleen homogenate would hasten the recovery of antibody production following exposure to X-rays.

Methods. Mice, males of an inbred NIH strain, were 11 to 13 weeks of age at the time of irradiation. Mice of another strain, (BALB/cAn X DBA₂J)F₁, hereafter referred to as BALB/c, were used in one experiment. Littermates and treatment groups were distributed as nearly uniformly as possible within each group of 10 mice during the radiation exposure. Irradiation was carried out with a 200 KVP X-ray unit operating at 20 ma, with 0.25 mm Cu and 0.51 mm Al added filtration. HVL equal to 0.76 mm Cu. Radiation dose was 450 r (LD₄, 28 days) and in one experiment, 525 r, given at about 55 r per minute. Within an hour or 2 after irradiation, groups

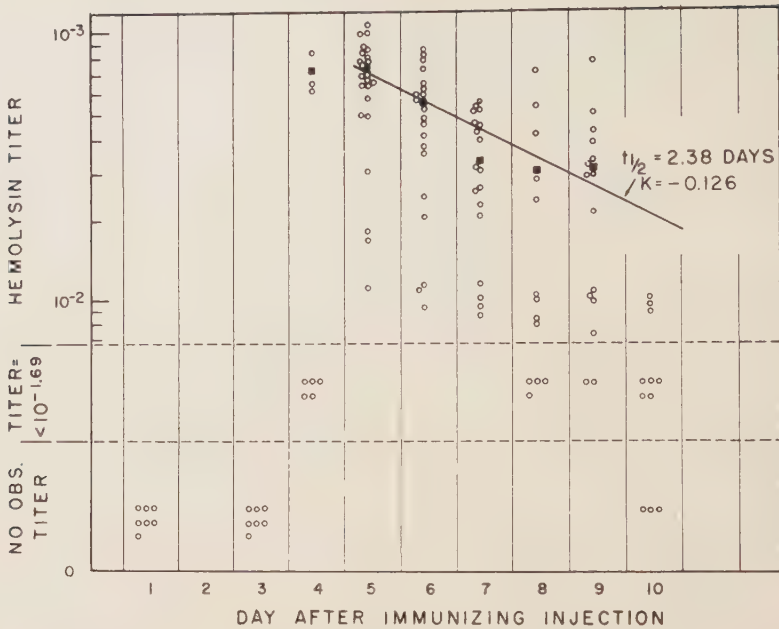


FIG. 1. Serum hemolysin titers of individual mice (○) sacrificed during the 10 day period following a single immunizing injection of sheep erythrocytes. Mean values for titers above 1:50 ($10^{-1.69}$) are denoted by (■), while titers below 1:50 or serums containing no observed hemolysin are shown schematically.

of the mice were given injections of suspensions of homologous spleen or bone-marrow from 4-week-old donor mice. Tissue, ground in a Potter type homogenizer and suspended in Tyrode's solution was injected intravenously. Each mouse received 0.5 ml of the suspension adjusted to contain the amount of tissue recoverable from the spleen or both femoral marrows of a single donor animal.

Mice were immunized with a single injection of a 2.5% suspension of washed sheep erythrocytes given intravenously at the rate of 0.01 ml per gram of body weight. This volume contained approximately 3.7×10^{11} sheep erythrocytes which, from preliminary trials, was the lowest concentration that would yield satisfactory hemolysin titers in control mice. Serum from the individual mice collected at sacrifice was glycerinized and stored at about 3°C until the titrations were done within 3 to 4 weeks after the collection. Serum hemolysin content was estimated colorimetrically for individual mice following the method described by Taliaferro(12). Preliminary experiments had to be done to determine both the amount of the peak titer and

the day upon which it would occur after a single immunizing injection. Experiments were then carried out to compare the peak serum hemolysin titers of irradiated homogenate-treated mice with their irradiated controls. In these experiments serum was collected on the 5th day after immunization from mice which had received their immunizing injection during the first or subsequent weeks through the 7th week after exposure to 450 r.

The possibility that the induction phase for the production of hemolysin might have been lengthened as a result of irradiation was tested in the irradiated homogenate-treated mice and their irradiated controls in other experiments. Serums of mice which had received their immunizing injection on the 4th, 5th, 6th or 7th week following exposure to 450 r were collected for hemolysin titration on the 5th or 6th through the 9th day after immunization.

Results. Initial phases of hemolysin production following a single injection of sheep erythrocytes are illustrated in Fig. 1, in which the titers are shown as the negative logarithms of the dilution per ml of the serum required

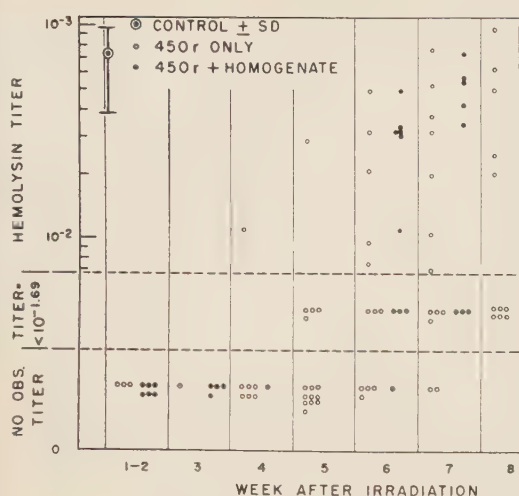


FIG. 2. Serum hemolysin titers on 5th day after immunization in marrow or spleen homogenate-treated irradiated mice compared with their irradiated controls. The mice were given a single immunizing injection of sheep erythrocytes during the indicated week following X-ray exposure and were sacrificed for the collection of their serums 5 days later.

to produce 50% hemolysis in a standard suspension of sheep erythrocytes. Serum samples in which the hemolysin content was just detectable, *i.e.* $<1:50$ or not detectable by our procedures, have been represented schematically in Fig. 1 and succeeding figures. The highest mean titer of the series, -2.73 , corresponding to a dilution of $1:537 \pm 333$ (S.D.) per ml, occurred on the 5th day for serums of 23 mice. The slope of the line fitted

by least squares through the means of titers above $1:50$ for days 5 through 10 has the value -0.126 . If it is assumed that group results reflect the changes in circulating hemolysin for any one animal, this slope represents a half life of about 2.4 days for the antibody.

Hemolysin titers on the 5th immunization day in spleen or marrow homogenate-treated mice are compared with their irradiated littermate controls in Fig. 2. Since no difference in hemolysin response was observed between the irradiated mice treated with bone marrow and those treated with spleen homogenate, the titrations of serums following these two treatments are given as for homogenate treatment. A gradual recovery of the immune response began during the 4th week after exposure to 450 r and continued at least into the 7th week (Fig. 2). The peak hemolysin titers in homogenate-treated mice agree closely with the titers of their irradiated controls and no indication of an accelerated return of the immune response is seen to be associated with the treatment.

Serums of irradiated or irradiated homogenate-treated mice showed little or no evidence of hemolysin production until the 4th week following exposure to 450 r. The homogenate treatment stimulated the early recovery of hematopoiesis since total leucocyte counts averaged significantly higher at 11 days after irradiation in 10 marrow and 10 spleen-treated mice than they did in 7 irradiated control mice

TABLE I. Hemolysin Production in BALB/c Mice Given Marrow or Spleen Homogenate after Exposure to 525 r.

	No. mice per group	1.5	Week following day of X-ray		
			3	4	5
Controls					
Hemolysin \pm S.E.*	2- 4		387 \pm 39	236 \pm 50	287
WBC $\dagger \pm$ S.E.	4	14.5 \pm 3.8		12.0 \pm 1	
Spleen wt (mg) \pm S.E. \ddagger	4		175 \pm 7.0	215 \pm 8.5	253 \pm 48.7
525 r only					
Hemolysin	9-10		---	60	<50
WBC	10	4.10 \pm .05	1.8 \pm .4	5.8 \pm .6¶	
Spleen wt	9-10		223 \pm 82	124 \pm 101.6	124 \pm 12.3
525 r + homogenate					
Hemolysin	15-18		---	56	60
WBC	19	7.6 \pm .5	7.9 \pm .4	8.5 \pm .6¶	
Spleen wt	17-19		130 \pm 8.4	208 \pm 23.4	188 \pm 21.9

* Dilution/ml of mouse serum giving 50% hemolysis in a standard suspension of sheep erythrocytes. † Total leucocytes $\times 10^3/\text{mm}^3$. ‡ Spleen wt in mg at sacrifice. § No observed serum hemolysin. || WBC on day 18. ¶ WBC on day 26.

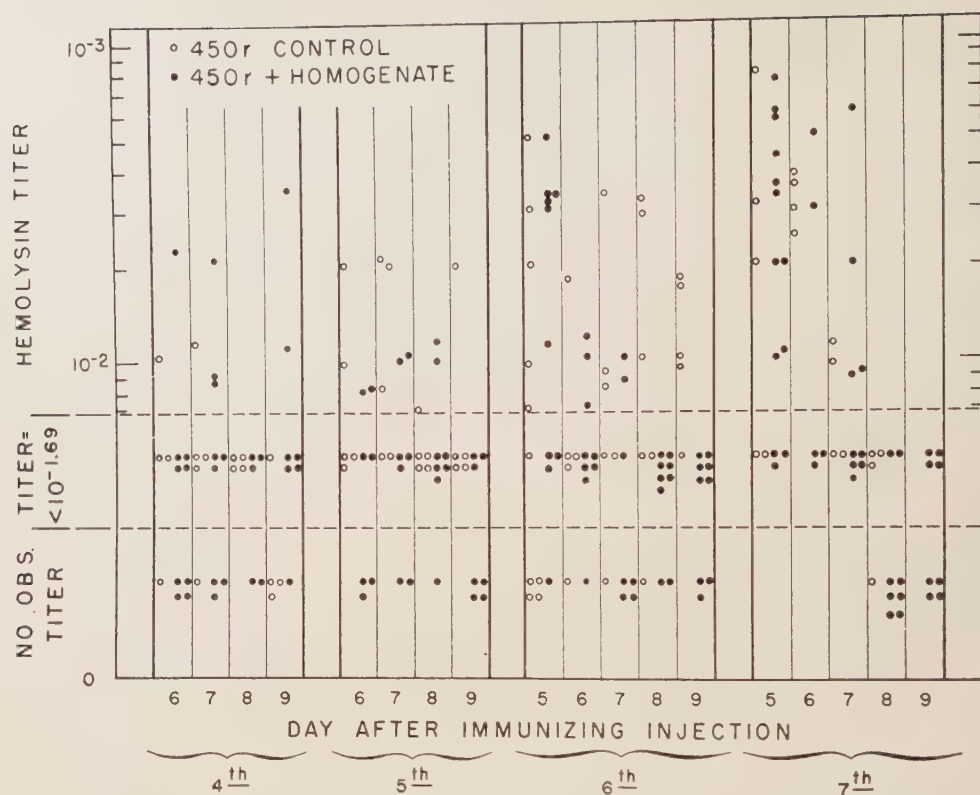


FIG. 3. Serum hemolysin titers in irradiated homogenate-treated mice and their irradiated controls for the indicated daily intervals during the 4th through 7th wk following exposure to 450 r.

(2145 ± 155 , 2531 ± 591 , 528 ± 110 cells per cu mm, respectively). Similar results were obtained in another experiment using BALB/c mice exposed to a somewhat higher radiation dose as shown in Table I. It is worthwhile to point out that the hyperplastic spleens seen in the third week (Table I) were not associated with improved hemolysin titers.

Fig. 3 summarizes the results of experiments which show that the time of maximum antibody production in the irradiated mice was not noticeably lengthened by the radiation exposure since there was no evidence that peak titers occurred later than the 5th day after immunization. The data of Fig. 3 indicate that the hemolysin titers tended to decline as the time from immunization increased, at least up to the 9th day. Furthermore, the homogenate treatment was without marked effect on the time of occurrence of maximum

titer during the 4th through the 7th week after exposure to 450 r. The gradual recovery of the immune response following irradiation is shown in the higher individual titers and the relatively smaller proportion of low titered serums in the 7th week compared to the 4th and 5th weeks (Fig. 3).

Discussion. Homologous bone marrow and spleen cell homogenates, injected shortly after X-ray, fail to hasten the recovery of the production of anti-sheep erythrocyte antibody in irradiated mice. The favorable effect of the homogenate treatment on survival after exposure to X-ray doses in the lethal range is attributable in part at least to its salutary effect on the blood-forming tissues. The marrow and nearly every organ, as well as specific cell type, including lymphocytes and plasma cells, have been at various times considered the site of antibody formation(13). Although

homogenates of marrow or spleen either furnish cells which repopulate the hematopoietic tissue damaged by the radiation or supply a humoral substance which hastens the recovery of hematopoiesis(14), they produce no marked effect on the mechanism of hemolysin synthesis when given just after irradiation. Independent studies(15) of the cellular composition of the spleen and marrow of mice after exposure to 450 r have not shown unusual proportions of mature plasma cells either at 21 days when the spleens are enlarged or at 35 days when the antibody response has begun to recover.

More or less normal immune responses have recently been obtained in irradiated rabbits given antigen that had been incubated with lymph node cells(6) or minced spleen(7). In the experiments described in this paper (Fig. 2) antigen given as early as 2 days after the homogenate and within 4 days of irradiation did not result in a hemolysin response.

Summary and conclusions. 1. Recovery of the production of hemolysin in irradiated mice receiving bone marrow or spleen cell homogenate occurred at the same rate as in litter-mate control mice exposed to 450 r of X-rays. 2. Recovery of antibody production in response to injected sheep erythrocytes is gradual and begins in the 4th week after exposure of mice to 450 r. Recovery of the hemolysin response is not complete even at 7 weeks after this dose of radiation since many of the mice had peak serum hemolysin titers that were below the limits of non-irradiated controls. 3. The time required for the development of

peak titer following a single immunizing injection was not lengthened by exposure to 450 r whole-body irradiation.

1. Hektoen, L., *J. Infect. Dis.*, 1915, v17, 415.
2. Taliaferro, W. H., and Taliaferro, L. G., *J. Immunol.*, 1951, v66, 181.
3. Hale, W. M., and Stoner, R. D., *Rad. Res.*, 1954, v1, 459.
4. Dixon, F. G., Talmage, D. W., and Maurer, P. W., *J. Immunol.*, 1952, v68, 693.
5. Jacobson, L. O., Robson, M. J., and Marks, E. K., *PROC. SOC. EXP. BIOL. AND MED.*, 1950, v75, 145.
6. Harris, T. N., Harris, S., and Farber, M. B., *Fed. Proc.*, 1954, v13, 496.
7. Jaroslow, B. N., and Taliaferro, W. H., Abstract of paper presented at 3rd Ann. Meet. of Rad. Res. Society, May 1955.
8. Jacobson, L. O., Simmons, E. L., Marks, E. K., Gaston, E. O., Robson, M. J., and Eldredge, J. H., *J. Lab. and Clin. Med.*, 1951, v37, 683.
9. Smith, W. W., Marston, R. Q., Ruth, H. J., and Cornfield, J., *Am. J. Physiol.*, 1954, v178, 283.
10. Lorenz, E., Congdon, C., and Uphoff, D., *Radiol.*, 1952, v58, 863.
11. Marston, R. Q., Ruth, H. J., and Smith, W. W., *PROC. SOC. EXP. BIOL. AND MED.*, 1953, v83, 289.
12. Taliaferro, W. H., and Taliaferro, L. G., *J. Infect. Dis.*, 1950, v87, 37.
13. The Nature and Significance of the Antibody Response, Symposium No. 5, N. Y. Acad. Sci., Columbia Univ. Press, 1953, 13.
14. Congdon, C. C., and Lorenz, E., *Am. J. Physiol.*, 1954, v176, 297.
15. Grenan, Marie M., unpublished observations from this laboratory.

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Anticonvulsant Properties of Procaine, Cocaine, Adiphenine and Related Structures.* (21979)

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In low concentrations, local anesthetics block conduction without depolarizing nerves, by stabilizing neuronal membranes(1,2); but large doses given systemically can induce convulsions. It was therefore of interest that Yasukata(3) reported that procaine exhibits anticonvulsant effects in rabbits. Among the numerous classes of chemicals capable of preventing experimentally-induced and/or clinical seizures, there are several antihistaminic agents(4-6), ethers of phenylacetic and phenylbutyric acids(7,8), and other closely related substances. For these reasons, it seemed worthwhile to attempt to disclose some effective structural denominator of anticonvulsant activity among compounds in which dialkylaminoalkyl is linked via O, H or C to an aryl or alkyl radical. To initiate this study, procaine and adiphenine (Trasentine) were tested for anticonvulsant properties in mice and compared with certain of their chemical and pharmacological congeners, particularly cocaine. The results obtained provide the basis for this report.

Methods. Male albino mice (CF #1 strain) obtained from the Carworth Farm were used as experimental animals. They were maintained on Purina Laboratory Chow and allowed free access to food and water except during the short time they were removed from their cages for testing. Anticonvulsant potency (ED₅₀) was determined by the maximal electroshock seizure (MES) test (50 mA alternating current, 0.2-second stimulus duration, corneal electrodes), and neurotoxicity (TD₅₀) was determined on the basis of overt signs of minimal neurological deficit. The de-

tails of the MES test and the endpoints employed for determination of acute neurotoxicity in mice have been described in full elsewhere(9). In order to determine the peak time for anticonvulsant action, the estimated ED₈₅ for each drug was administered intraperitoneally to several groups of 6 mice each. Each group was then subjected to the MES test after a different time interval and the number of animals protected (*i.e.*, failing to exhibit the tonic-extensor component) was recorded for each group. The results obtained were plotted on graph paper and the time of peak effect estimated from the plotted points. For the determination of the ED₅₀ (or TD₅₀), groups of 10 mice were given various doses of the experimental drug and tested at the previously determined time of peak effect until at least 3 points were established between complete protection (or toxicity) and no protection (or toxicity). The results were then plotted on logarithmic probability paper and a regression line was visually fitted to the plotted points. From this plot of the data, the ED₅₀ or TD₅₀ was determined and 95% confidence limits were calculated by the method of Litchfield and Wilcoxon(10). The drugs subjected to the MES and neurotoxicity tests were as follows: procaine hydrochloride, cocaine hydrochloride, adiphenine (Trasentine),‡ β -(*o*-benzylphenoxy)- α -diethylaminoethane (No. BL 338-22)§, 2-diethylaminoethyl-1-phenylcyclopentane-1-carboxylate hydrochloride (caramiphen, Parpanit), diethylaminoethanol, and diethyl-(2-hydroxyethyl)-methyammonium bromide- α -phenylcyclohexaneglycolate (Ba 5473, oxyphenonium, Antrenyl).‡ The drugs were administered intraperitoneally as aqueous solutions. All animals were observed for the effect of the drugs on

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§ Supplied by Dr. S. B. Binkley, Bristol Laboratories.

TABLE I. Neurotoxicity and Anti-electroshock Potency of Procaine, Cocaine, and Trasentine.*

Drug	Neurotoxicity (TD ₅₀), mg/kg	Max electroshock seizure test†	
		Effective dose (ED ₅₀), mg/kg	Protective index (P.I.)
Procaine	105 (98-113)	45 (42-49)	2.3 (2.1-2.5)
Cocaine	32 (29-36)	17.5 (15-23)	1.8 (1.6-2.0)
Trasentine	123 (116-131)	62 (58-67)	2.0 (1.8-2.2)

* All values in parentheses indicate 95% confidence limits.

† 50 mA, 0.2-sec. alternating current, corneal electrodes.

postictal phenomena, the details of which have been described elsewhere(11,12). In addition, the ED₅₀, as determined by the MES test, and the TD₅₀, as determined by the neurotoxicity test, for procaine hydrochloride, cocaine hydrochloride, and Trasentine were administered to groups of 5 mice each and tested for ability to prevent the tonic-extensor component of maximal seizures induced by the rapid intravenous injection of 38 mg/kg of pentylenetetrazol (Metrazol) (MMS test). The details of the MMS test have been published in full elsewhere(13).

Results. Procaine, cocaine, and Trasentine proved to possess anticonvulsant action, but their antiseizure activity appeared and disappeared very quickly. The time of peak action was estimated to be 5 minutes; by 20 minutes little if any residual effect could be observed. Therefore, all tests were performed 5 minutes after drug administration.

The neurotoxicity and anti-electroshock potency for procaine, cocaine, and Trasentine are summarized in Table I from which it may be seen that cocaine is 3 to 4 times more toxic than procaine or Trasentine. Indeed, 32 mg/kg of cocaine produced signs of minimal neurotoxicity in 50% of mice as compared to 105 mg/kg for procaine and 123 mg/kg for Trasentine. The initial signs of cocaine neurotoxicity were agitation and increased excitability; larger doses caused ataxia and postural abnormalities. The initial signs of procaine and Trasentine neurotoxicity were postural abnormalities and abnormal gait, especially

dragging of the hindlimbs; moderately intoxicated animals were unable to maintain equilibrium. Doses of procaine in excess of 120 mg/kg produced excitation.

All 3 agents were capable of preventing the hindleg tonic-extensor component of maximal electroshock seizures (MES). Cocaine was approximately 2½ times more potent than procaine in this regard, and 3½ times more potent than Trasentine. For example, 17.5 mg/kg of cocaine abolished the hindleg tonic-extensor component in 50% of mice, whereas 62 mg/kg of Trasentine were required to produce the same level of protection. Larger doses than those required to protect 50% of animals by the MES test not only abolished the tonic-flexor phase as well as the tonic-extensor phase, but also produced the "anticoma effect" in the postictal period (prevention of immediate postseizure depression). Approximately 50% of animals receiving 75, 27.5, and 80 mg/kg of procaine, cocaine, and Trasentine, respectively, manifested this anticoma effect.

The protective index, *i.e.*, the ratio between TD₅₀ and ED₅₀, was remarkably similar for all 3 agents. For example, the P. I. for cocaine was 1.8, whereas that for procaine was 2.3 and that for Trasentine was intermediate to these two values. Thus, all three agents exhibited anticonvulsant activity in non-toxic doses.

The anti-Metrazol activity exerted by the TD₅₀ and the ED₅₀ of each of the three agents was measured by the MMS test. The results obtained are summarized in Table II from which it may be seen that all 3 agents have the ability to decrease the incidence of the

TABLE II. Anti-Metrazol Effect of Procaine, Cocaine, and Trasentine.

Drug	Dose, mg/kg	Prevention of tonic-extensor component	Mortality
Procaine	45 *	3/5	4/5
	105 †	5/5	5/5
Cocaine	17.5*	3/5	2/5
	32 †	3/5	3/5
Trasentine	62 *	2/5	4/5
	123 †	4/5	2/5

* ED₆₀ as determined by MES test.

† TD₅₀ " " " neurotoxicity test.

tonic-extensor component of maximal Metrazol seizures; however, the required doses frequently synergized with the convulsant drug to cause death. The clonic component of Metrazol-induced maximal seizures was not abolished even by toxic doses of the 3 agents.

Beta - (o-benzylphenoxy)- α -diethylaminoethane (No. BL 338-22) and Parpanit, the chemical structures of which comply with the prerequisites mentioned in the introduction, were also tested for neurotoxicity and for ability to modify the pattern of maximal electroshock seizures. Their time of peak anticonvulsant action was estimated to be 10 minutes; by 30 minutes little if any residual effect could be observed. Both compounds were anticonvulsant, but their protective indices were near unity. Thus, the ED₅₀s for No. BL 338-22 and Parpanit were 52 and 80 mg/kg, respectively; their P.I.s were 1.0 and 1.5, respectively. In contrast, diethylaminoethanol and Antrenyl, the chemical structures of which only partially comply with the previously described structural prerequisites, were ineffective by the MES test even in toxic doses.

Discussion. The present investigation demonstrates that anticonvulsant activity is embodied not only in procaine, as was first reported by Yasukata(3) in rabbits, but also in cocaine and Trasentine. Since procaine and cocaine in large toxic doses are convulsant, it is of particular interest that in non-toxic doses these drugs act as anticonvulsants.

The anticonvulsant action of procaine, cocaine and Trasentine is rapid in onset and brief in duration. Peak action was observed 5 minutes after drug administration; by twenty minutes all anticonvulsant effect had disappeared. This very rapid onset of anticonvulsant activity and the evanescent duration of action probably account for previous failures to demonstrate antiseizure activity in local anesthetics.

The 5 compounds which have within their chemical structure a dialkylaminoalkyl linked via O, N or C to an aryl or aralkyl radical were all found to possess anticonvulsant activity as measured by the MES test; in contrast, the diethylaminoethanol moiety alone and Antrenyl, a quaternary ammonium com-

pound, were devoid of such activity. (The ability of prior administration of diethylaminoethanol, the hydrolytic product of procaine, to prevent procaine convulsions in experimental animals(14) is probably accounted for by competitive inhibition on the basis of similarity of chemical structure.) Although the above findings suggest that compounds within this chemical series share a common anticonvulsant nucleus, further studies will be necessary precisely to determine what specific structural elements are essential for antiseizure activity.

Procaine, cocaine, and Trasentine all exhibit anticonvulsant effects in the laboratory which are qualitatively similar to those produced by the therapeutically useful antiepileptic drugs. However, the potential clinical value of compounds of this class must be considered minimal because of the very short duration of action and relatively low protective indices. Nevertheless, the data presented suggest that a search for congeners with less fugitive action is warranted. It is of interest in this connection that Bernhard and Bohm (15) have demonstrated that intravenous injection of the local anesthetic lidocaine (Xylocaine), an aminoacyl amide, can modify electrically induced cortical discharges in cats and interrupt epileptic seizures in patients. Preliminary experiments in our laboratory indicate that this compound, in non-toxic doses, is highly effective in abolishing the tonic-extensor component of maximal electroshock seizures. Indeed, others(16) have observed that intravenous procaine paradoxically increases the threshold for electrically evoked cortical responses in rabbits, but causes repetition and increased voltage of such responses as well as widespread irradiation. Since it is likely that the small neurones in the brain are the earliest to be blocked after systemic administration of local anesthetics, the possibility exists that the seizures induced by this class of drugs may be due to selective depression of inhibitory systems(16).

Summary. Five compounds—procaine hydrochloride, cocaine hydrochloride, adiphenine (Trasentine), β -(o-benzylphenoxy)- α -diethylaminoethane, and Parpanit—the chemical structures of which have a dialkylaminoalkyl

linked via O, N or C to an aryl or aralkyl radical, were tested in mice for neurotoxicity and for ability to modify maximal electroshock seizures. In addition, procaine, cocaine, and Trasentine were tested for ability to modify maximal Metrazol seizures and to prevent postictal depression (anticoma effect). For comparison, two compounds (diethylaminoethanol and Antrenyl), the chemical structures of which only partially comply with the above structural prerequisites, were tested only for neurotoxicity and for ability to modify maximal electroshock seizures. The results obtained may be summarized as follows: 1. Procaine, cocaine, and Trasentine prevent the tonic-extensor component of maximal electroshock seizures in non-toxic doses and exert an anticoma effect in doses near the toxic level. All 3 agents prevent the tonic-extensor component of maximal Metrazol seizures, but only in doses which frequently synergize with the convulsant to cause death. 2. All compounds tested, the chemical structure of which contains a dialkylaminoalkyl linked via O, N or C to an aryl or aralkyl radical, were found to exhibit anticonvulsant activity. In contrast, diethylaminoethanol and Antrenyl were devoid of anticonvulsant activity. 3. The data indicate that compounds within this series share a common anticonvulsant nucleus, but further studies will be necessary precisely to characterize its structural requirements. 4. The laboratory search for congeners with less transient anti-

convulsant action and higher protective index is warranted.

1. Bishop, G. H., *J. Cell. and Comp. Physiol.*, 1932, v1, 177.
2. Bennett, A. L., and Chinburg, K. G., *J. Pharmacol. and Exp. Therap.*, 1946, v88, 72.
3. Yasukata, M., *J. Yonago M. Assn.*, 1955, v6, 72.
4. McGavack, T. H., Elias, H., and Boyd, L. J., *Am. J. M. Sc.*, 1947, v213, 418.
5. Churchill, J. A., and Gammon, G. D., *J.A.M.A.*, 1949, v141, 18.
6. Swinyard, E. A., Jolley, J. M., and Goodman, L. S., *Proc. Soc. Exp. Biol. and Med.*, 1950, v75, 239.
7. Swinyard, E. A., Madsen, J. A., and Goodman, L. S., *J. Pharmacol. and Exp. Therap.*, 1954, v111, 54.
8. Sakai, S., Mishima, O., and Yuasa, Y., *J. Yonago M. Assn.*, 1954, v5, 47.
9. Swinyard, E. A., Brown, W. C., and Goodman, L. S., *J. Pharmacol. and Exp. Therap.*, 1952, v106, 319.
10. Litchfield, J. T., Jr., and Wilcoxon, F., *ibid*, 1949, v96, 99.
11. Frommel, E., and Radouco-Thomas, C., *Helv. Physiol. Pharmacol. Acta.*, 1953, v11, 270.
12. Tanaka, K., in press, 1955.
13. Goodman, L. S., Grewal, M. S., Brown, W. C., and Swinyard, E. A., *J. Pharmacol. and Exp. Therap.*, 1953, v108, 168.
14. Richards, R. K., and Kueter, K. E., *J. Pharmacol. and Exp. Therap.*, 1946, v87, 42.
15. Bernhard, C. G., and Bohm, E., *Experientia*, 1954, v10, 474.
16. Toman, J. E. P., and Davis, Jean P., *Pharmacol. Rev.*, 1949, v1, 425.

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Reserpine and Thyroid Function. (21980)

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The introduction of reserpine with its mild sedative effects into common clinical use raises the question of the influence of this agent on thyroid function. Reserpine is generally assumed to act upon certain medullary centers(1), but the experiments of McQueen *et al.*(2) and of Kuschke and Gruner(3) appear to indicate a direct peripheral action as well. Very little work has been reported in the literature which would indicate whether reserpine exerts any influence either directly on the thyroid gland, or, through the hypothalamic-pituitary-thyroid axis, upon thyroid function. Certain observations, particularly the reserpine-induced bradycardia(4) and the effect of reserpine upon weight gain in "constitutional leanness"(5) could indicate an anti-thyroid activity of the drug. Moreover, Kuschke and Gruner(3) have reported that in the rat the effect of parenterally administered thyroxine upon the BMR is prevented by the administration of reserpine in large doses. However, the two published reports on the effect of Rauwolfia preparations on thyroid function as measured by the basal metabolic rate agree in finding no consistent effect. Erban and collaborators(6) found no effects in rats with a number of commercial preparations, and Moyer and associates(7) found no consistent changes in a small series of patients. Moyer also studied his patients by means of a radioiodine uptake test and obtained no consistent changes due to 6 weeks' medication with several crude Rauwolfia preparations. Since many patients entering thyroid clinics give a history of treatment with Rauwolfia preparations, it was considered important to establish whether this drug exerted any appreciable influence upon any of the commonly used criteria of thyroid function. A study was there-

fore undertaken in 18 chronically ill patients receiving reserpine at a level commonly used for the reduction of blood pressure.

To reconcile our results with those of Kuschke and Gruner the effect of reserpine in very high doses on thyroid function was also studied in a small number of rats.

Methods. Eighteen male patients were included. Blood pressure was determined daily throughout the study period. Base line values were obtained for the following criteria of thyroid function: (a) Serum total cholesterol, determined by the method of Kanter, Goodman and Yarborough(8); (b) 24-hour thyroidal radioiodine uptake; (c) 24-hour urinary radioiodine excretion; (d) serum protein-bound stable iodine, determined by Barker's (9) method; and (e) the 24-hour thyroxine synthesis measured according to Morton(10). In addition, the effect of a standard dose of adrenaline upon the level of circulating eosinophiles was determined by Thorn's(11) method. After completion of the base-line studies all patients received reserpine (Serpasil-Ciba) at a level of 0.125 mg t.i.d. for 2 days. The dose was then raised to 0.25 mg t.i.d. for another 24 days. In 2 patients the dose had to be reduced to 0.125 mg t.i.d. again after 1 and 11 days at the higher level, respectively, because of side effects. On the last 2 days of drug administration the battery of thyroid function and Thorn tests were repeated.

In the animal experiment 6 male Sprague-Dawley rats were injected intravenously with 0.40 mg/kg of serpasil in solution 2 hours before tracer iodine administration, and then received 0.60 mg/kg by subcutaneous injection every 3 hours. Four control rats received similar volumes of the vehicle furnished by Ciba Pharmaceuticals at the same time. Twenty-four hour thyroidal radioiodine uptake was determined and serum hormonal iodine synthesis were measured in all animals by the methods of Morton(10) and of Ingbar (12)

* The Radioiodine used in this investigation was supplied by the Oak Ridge National Laboratory on allocation from the U. S. Atomic Energy Commission.

The authors wish to express their gratitude to Miss M. Whitney, R.N., for invaluable help in this study.

TABLE I. Post-Serpasil Thyroid Parameters as % of Pre-Treatment Level with Standard Deviations.

Group	No.	Cholesterol	24-hr I ¹³¹ uptake	PBI	Urinary excretion*	Tx/To†
Whole	18	101 ± 22	96 ± 54	112 ± 16	111 ± 43	92 ± 41
+++	8	102 ± 28	110 ± 73	113 ± 24	118 ± 50	99 ± 46
++	7	99 ± 16	90 ± 34	110 ± 8	99 ± 23	77 ± 34
±	3	101 ± 16	67 ± 6	115 ± 13	120 ± 73	135 ± 19

+++ = Decrease of 20% or more in the mean systolic blood pressure. ++ = Moderate decrease in blood pressure. ± = Doubtful effect on blood pressure.

* 24 hr urinary excretion of radioiodine.

† % of 24 hr serum I¹³¹ activity present as hormonal iodine.

Results. A. Effects on Thyroid Function. Table I summarizes the results obtained with our group of patients, most of whom were markedly hypertensive prior to therapy. All patients participating in this study were judged to be euthyroid initially from the PBI and radioiodine uptake values. However, the thyroxine synthesis values were below 2% in $\frac{2}{3}$ of the patients, which represents the lower limit of the euthyroid range. On recalculating the data by Ingbar's method, 10 patients had serum radio-thyroxine levels below .008% of the administered dose per liter of plasma, and only one patient showed a value above .012% per liter. In previous studies we have found low thyroxine synthesis values in the presence of normal PBI and uptake values in patients with cardiac disease of long duration (13), and we believe this to be a consequence of chronic stress. In Table I the experimental group has also been subdivided according to the effect upon the blood pressure produced by reserpine. Since the blood pressure was quite labile in most of the subjects, the patients were grouped into three categories according to the mean drop in blood pressure produced, with +++ indicating a good response (>20% drop of the mean systolic pressure); ++ a fair one and ± a doubtful or insignificant change due to the drug. The variability of the results may be related to the hypertensive state of most of these patients, since equally varied and random changes were found in two hypertensive subjects who were merely observed during the experimental period without medication, and since similar variability of the I¹³¹ uptake and BMR have been reported by Moyer(7).

From the results it is clear that reserpine at

the levels employed does not affect thyroid function significantly.

B. Effects on the Thorn test. The Thorn test with adrenaline is not universally accepted as a test of the functional state of the hypothalamic-pituitary-adrenal axis. However in healthy individuals a drop of more than 30% of the circulating eosinophiles is produced 4 hours after an injection of 0.2 mg of adrenaline subcutaneously. In only 3 patients out of 14 was a drop of less than 30% observed in the number of circulating eosinophiles before reserpine therapy. After administration of reserpine for 25 days, 11 out of 17 patients showed no significant eosinopenic response. While the Thorn test with adrenaline certainly is not a specific test for pituitary-adrenal function, the reversal of the test under the influence of reserpine is of interest. At least part of the eosinopenic effect of adrenaline is generally assumed to be mediated via effects on cerebral centers higher than the pituitary. Since reserpine depresses afferent impulses to certain subcortical centers(14) and lowers central sympathetic reactivity(15) the results obtained in this study agree with the generally accepted mode of action of reserpine. However, the mechanism by which adrenaline exerts its eosinopenic effect is so poorly understood that it is not permissible to speculate on this basis alone.

C. Effects of reserpine at high dose levels. Since Kuschke and Gruner had observed what appeared to be an antagonism between reserpine and thyroxine in the rat, it became of interest to investigate whether any anti-thyroid activity of reserpine might occur in this species. Employing the drug at low levels of .10 mg/kg/day, the results were similar to those

TABLE II. Thyroid Activity of Rats on High Doses of Serpasil.

Group	No.	24 hr uptake (%)	Tx/To	Tx, % AD/1*
Control	4	8.4 ± 2.1	57.2 ± 10.0	.088 ± .018
Serpasil	6	23.3 ± 4.1	10.8 ± 5.2	.18 ± .07

* Serum thyroxine- I^{131} as % of administered I^{131} dose/l of serum.

obtained in man, with neither the 24-hour uptake nor the thyroxine synthesis significantly affected. It was then decided to use reserpine at a dose level which had been shown to raise significantly the arousal in the brain stem of the rat(16). Under the conditions specified the data in Table II were obtained. It is significant that the reserpine-treated animals showed the symptoms of hypotensive shock: nasal congestion, somnolence and ptosis, as well as diarrhea. There was an obvious interference with renal excretion of iodide: Serum inorganic radioiodide levels were 20 times those in the controls 24 hours after tracer administration. This explains both the higher thyroidal uptake and the higher thyroxine synthesis value as calculated by Ingbar's method. It is possible that poor absorption of peripherally administered thyroxine due to the reserpine-induced hypotension also explains Kuschke and Gruner's results.

Summary. Reserpine at the dose levels employed clinically for the treatment of hypertension does not affect thyroid function in man. The effects of large doses in the rat appear to be due to hypotensive effects and in-

terference with the renal excretion of iodide.

1. Bhargava, K. P., and Borison, H. L., *Fed. Proc.*, 1955, v14, 319.
2. McQueen, E. G., Doyle, A. E., and Smirk, F. H., *Nature*, 1954, v174, 1015.
3. Kuschke, H. T., and Gruner, H., *Klin. Wochenschr.*, 1954, v32, 563.
4. Plummer, A. T., Earl, A., Schneider, J. A., Trapold, J., and Barrett, W., *Ann. N. Y. Acad. Sci.*, 1954, v59, 8.
5. Genest, T., Adamkiewicz, L., Robillard, R., and Tremblay G., *Canad. Med. Assn. J.*, 1955, v72, 490.
6. Erban, W., Lindner, A., and Watschinger, B., *Sci. Pharm.*, 1954, v22, 145.
7. Ford, R. V., Livesay, W. R., Miller, S. I., and Moyer, J. H., *Med. Rec. and Ann. Texas*, 1953, v47, 608.
8. Kanter, S. L., Goodman, J. R., and Yarborough, J., *J. Lab. Clin. Med.*, 1952, v40, 303.
9. Barker, S. B., Humphrey, M. J., and Soley, M. H., *J. Clin. Invest.*, 1951, v30, 55.
10. Morton, M. E., *Calif. Med.*, 1953, v78, 277.
11. Recant, L., Hume, D. M., Forsham, P. H., and Thorn, G. W., *J. Clin. Endocrin.*, 1950, v10, 187.
12. Ingbar, S. H., Freinkel, N., Hoeprich, P. D., and Athens, J. W., *J. Clin. Invest.*, 1954, v33, 388.
13. Morton, M. E., Goodman, J. R., and Florsheim, W. H., unpublished results.
14. Meier, R., Bein, H. J., Gross, F., Tripod, J., and Tuchmann-Duplessis, H., *Comp. Rend. Acad. Sci.*, 1954, v238, 961.
15. Schneider, R. A., *Ann. N. Y. Acad. Sci.*, 1954, v61, 150.
16. Barraclough, C. A., and Sawyer, C. W., personal communication.

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Electrolyte and Water Composition of Muscle and Liver in Hereditary Obese-Hyperglycemic Syndrome of Mice.* (21981)

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(Introduced by A. Baird Hastings.)

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The hereditary obese-hyperglycemic syndrome of mice(1) has, among other characteristics, an insulin resistant hyperglycemia and glycosuria(2). In a small proportion of obese animals, the hyperglycemia is not present but can be immediately elicited by injection of a single dose of growth hormone to which obese animals are extremely sensitive (3). Non-obese animals show the usual resistance of rodents to the diabetogenic effect of growth hormone. The syndrome is transmitted as a Mendelian recessive trait which affects both sexes in a similar manner. A number of relationships have been recognized between potassium deficiency and carbohydrate metabolism. The abnormalities of glucose production and utilization observed in insulin deficient rat liver slices incubated at a potassium concentration optimal for glycogenesis were similar to those observed in normal slices incubated in a low potassium medium(4,5,6).[‡] Although no abnormality of potassium concentration was demonstrated in the liver cells of intact insulin-deficient rats (8), hyperglycemia and depletion of liver glycogen have been reported in rats maintained on a potassium deficient diet(9). Fatty acid metabolism of rat liver slices was increased by a high potassium concentration in the incubation medium(10).

These observations suggested that tissue analyses for water, fat potassium, sodium and chloride of mice with the hereditary obese-hyperglycemic syndrome and the estimation

of intracellular concentrations of potassium and sodium might be of interest.

Materials and methods. Six male and 3 female mice with the hereditary obese-hyperglycemic syndrome were used. Three male and 3 female litter-mates served as controls. After a 24-hr fast (water *ad lib.*) the animals were anesthetized by intraperitoneal injection of one per cent aqueous sodium amytal. A dose of 0.2 mg per gram gave satisfactory anesthesia in 15 minutes. One obese animal required 0.3 mg per g. Blood sugar levels (11, 12) were determined on 0.02 ml of blood obtained from the tail. Blood for measurement of hemoglobin concentration was obtained in a similar manner. Serum for chloride(13) and sodium(14) determinations was separated from blood which was aspirated from the right side of the heart and placed under oil. Samples of muscle from the thigh and calf were dissected as free from adipose tissue as possible. Liver samples were obtained using care to avoid the gall bladder. Both tissue samples were minced, dried, defatted and extracted with 0.75 N nitric acid(15). Duplicate samples were used to measure the blood content of the tissues(15). The chloride content of the extracts was determined by a modification of the colorimetric micro-method of Lowry and co-workers(13). The sodium and potassium contents were determined by flame photometry(14).

Results. In Tables I and II the data from obese and control animals have been compared, all values in the latter table being calculated on a blood-free-fat-free basis. The obese animals were significantly hyperglycemic when sampled in the fed state several days before being sacrificed. The serum chloride concentration was lower in the obese animals, but this would only partially account for the difference in liver chloride concentration observed. The dry fat-free weight of

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[‡] The *in vivo* intracellular potassium concentration can be maintained when liver slices are incubated only by increasing the potassium concentration of the medium to a level far higher than that of extracellular fluid(7).

TABLE I. Mean Body Weight, Blood and Serum Values.

	No. used	Age (days)	Body wt (g)	Blood		Serum	
				Fasting sugar (mg %)	Normal sugar (mg %)	Na (mEq/l)	Cl (mEq/l)
Obese	9	133	62 ± 7	151 ± 33	218 ± 38	145.6 ± 1.9	101.0 ± 1.8
Control	6	137	28 ± 2	94 ± 17	106 ± 13	146.3 ± .8	106.0 ± .9
p value			<.01	>.1	<.05	>.1	<.05

Digits following the ± sign are stand. error of the mean.

muscle was higher per unit of wet fat-free tissue in the obese animals. The potassium content of muscle was significantly lower per unit of dry fat-free tissue in the obese mice.

In Table III a correction has been applied to the data for the increment of water injected with the sodium amytal used for anesthesia. Derived values have been calculated for the amount of extracellular water in muscle, assuming that all chloride was extracellular(15). This permitted calculation of the concentration of potassium and sodium in intracellular water. No significant differences in these derived data between the obese and control mice were observed. The column headed " $(H_2O)_E$ less adipose tissue" will be discussed later.

Derived values have been calculated for the amount of extracellular water in liver, assuming that all the sodium was extracellular(16). This permitted calculation of the concentration of potassium and chloride in intracellular water. Here, as in muscle, no significant differences between the two groups were observed.

Discussion. Correction of the data for the increment of water injected with sodium amytal was necessary before comparing obese with

control mice (Table III) because this increment was proportionally greater in the obese animals. With one exception the water injected was proportional to body weight, but the obese group contained less water per unit of body weight than their controls. To apply such a correction the following relationship obtained on mice with the same abnormality by Mayer and Hagman was used(17):

Grams of Total Body Water = $12 + (\text{Grams of Body Weight} \times 0.11)$. Data were also reported by these authors showing that 15 minutes after intraperitoneal injection of deuterium oxide, the volume of dilution for the isotope labeled water had reached 88% of its equilibrium volume. These findings permitted calculation of the approximate water content of cells and extracellular fluid prior to the injection of aqueous sodium amytal solution. Specifically, the original serum water and tissue water values were multiplied by the following factor:

Total Body Water

Total Body Water + (Injected Water \times 0.88)

Application of this factor to the data increased the difference between the two groups with re-

TABLE II. Mean Values for Muscle and Liver; Original Data. The values are reported per kilo of wet fat-free, blood-free tissue or of dry fat-free, blood-free tissue.

	Per kg wet fat-free tissue					Per kg dry fat-free tissue
	Dry fat-free wt (kg)	Wt of fat (kg)	Cl (mEq)	Na (mEq)	K (mEq)	K (mEq)
Muscle:						
Obese	.244 ± .002	.202 ± .041	14.4 ± .7	23.9 ± 1.1	108.4 ± 1.5	445 ± 6
Control	.235 ± .002	.075 ± .011	15.7 ± .7	23.7 ± .9	111.3 ± .9	474 ± 6
p value	<.01	<.05	>.1	>.1	>.1	<.01
Liver:						
Obese	.267 ± .002	.145 ± .021	26.8 ± .9	35.0 ± 1.3	84.7 ± 1.4	318 ± 6
Control	.259 ± .006	.103 ± .019	31.6 ± 2.0	39.1 ± 2.6	85.5 ± 2.5	329 ± 6
p value	>.1	>.1	<.05	>.1	>.1	>.1

Digits following the ± sign are stand. error of the mean.

TABLE III. Mean Values for Muscle and Liver Corrected for Water Injected with Anesthetic. Values are reported per kilo of wet fat-free, blood-free tissue or of intracellular water.

	Per kg wet fat-free tissue			Per kg intracellular water		
	Dry fat-free wt (kg)	Wt of fat (kg)	(H ₂ O) _E (kg)	(H ₂ O) _E less adipose tissue (kg)	(H ₂ O) _O (kg)	
Muscle:						
Obese	.256 ± .002	.212 ± .043	.133 ± .008	.107 ± .007	.612 ± .008	
Control	.241 ± .002	.077 ± .011	.138 ± .006	.129 ± .006	.622 ± .006	
p value	<.01	>.01	>.1	<.05	>.1	
Liver:						
Obese	.279 ± .001	.152 ± .022	.228 ± .007		.493 ± .008	5.3 ± 1.3
Control	.265 ± .007	.105 ± .019	.256 ± .018		.479 ± .012	6.9 ± 1.3
p value	<.1	>.1	>.1		>.1	>.1

Digits following the ± sign are stand. error of the mean.

Symbols: (H₂O)_E extracellular water; (H₂O)_O intracellular water; K_O intracellular potassium; Na_O intracellular sodium; Cl_O intracellular chloride.

spect to the dry fat-free weight of both tissue (muscle "p value" <0.01, liver "p value" <0.1). The serum chloride difference recorded in Table I was halved when the correction was applied.

It was apparent on dissection and confirmed by data in Tables II and III that considerably more adipose tissue was mingled with striated muscle fibres in the obese than in the control muscle samples. Data have been collected by Lowry and Hastings showing that adipose tissue from rat omentum contained 83% fat and 10% extracellular water(18). Assuming similar findings for adipose tissue of the obese and control mice, values were derived for (H₂O)_E of muscle fibres after deducting the fraction associated with adipose tissue in the muscle samples (Table III). This approximation of the "true" (H₂O)_E for muscle fibres gave a significantly lower value in the obese mice. The potassium concentration per unit of dry fat-free muscle was 6% lower in the obese group (Table II). However, (H₂O)_C per unit of dry fat-free muscle was 7% lower in the fat animals after correction for injected water. "(H₂O)_E less Adipose Tissue" was 22% lower when expressed per unit of dry fat-free muscle. This dehydration occurred even though water was available throughout the fasting period.

Studies in man have shown that the hyperglycemia and glycosuria following hypertonic glucose infusion is associated with sodium diuresis and a lesser degree of potassium diuresis(19). One may presume that the same type of electrolyte losses occurred in the obese mice and that while in the fasting state, dehydration was necessary to preserve the osmolarity of body fluids.

Summary. 1. Samples of striated muscle and liver from fasted mice with the hereditary obese-hyperglycemic syndrome were analyzed for water, fat, potassium, sodium, and chloride. 2. Some degree of intracellular dehydration and a greater degree of extracellular dehydration was observed without change in concentration of intracellular potassium.

1. Mayer, J., *Physiol. Rev.*, 1953, v33, 472.

2. Mayer, J., Bates, M. W., and Dickie, M. M., *Science*, 1951, v113, 746.

3. Mayer, J., Andrus, S. B., and Silides, D. J.,

Endocrinology, 1953, v53, 572.

4. Hastings, A. B., Teng, C. T., Nesbett, F. B., and Sinex, F. M., *J. Biol. Chem.*, 1952, v194, 69.

5. Renold, A. E., Teng, C. T., Nesbett, F. B., and Hastings, A. B., *ibid.*, 1953, v204, 533.

6. Hastings, A. B., Renold, A. E., and Teng, C. T., *Trans. Assn. Am. Phys.*, 1953, v67, 129.

7. Flink, E. B., Hastings, A. B., and Lowry, J. K., *Am. J. Physiol.*, 1950, v163, 598.

8. Tobian, L., Morse, W. I., and Hastings, A. B., unpublished.

9. Gardner, L. I., Talbot, N. B., Cook, C. D., Berman, H., and Uribe, C. R., *J. Lab. and Clin. Med.*, 1950, v35, 592.

10. Geyer, R. P., Meadows, M. F., Marshall, L. D., and Gongaware, M. S., *J. Biol. Chem.*, 1953, v205, 81.

11. Nelson, N., *ibid.*, 1945, v160, 61.

12. Somogyi, M., *ibid.*, 1945, v160, 61.

13. Lowry, O. H., Roberts, N. R., Leiner, K. Y., Wu, M-L., and Farr, A. L., *ibid.*, 1954, v207, 1.

14. Flame photometer, model 52A, modified. The Perkin-Elmer Corporation, Glenbrook, Conn.

15. Lowry, O. H., and Hastings, A. B., *J. Biol. Chem.*, 1942, v143, 257.

16. Lowry, O. H., Hastings, A. B., McCay, C. M., and Brown, A. N., *J. Gerontol.*, 1946, v1, 345.

17. Mayer, J., and Hagman, N. C., *PROC. SOC. EXP. BIOL. AND MED.*, 1953, v82, 647.

18. Lowry, O. H., and Hastings, A. B., unpublished observations.

19. Seldin, D. W., and Tarail, R., *Am. J. Physiol.*, 1949, v159, 160.

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Local Effects on the Feather Papilla of Thyroxine and of Progesterone.* (21982)

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Shedding and renewal of plumage occurs in birds in well-defined association with sexual and seasonal events. Normally, the standing lifeless feather is gradually expelled from the mouth of the follicle as a new feather arises following an activation which is initiated in the resting papilla, an organ that uninterruptedly occupies the internal well of the follicle and which is the actual site of the externally manifest cyclical events. Seasonal inertia of the papilla is a familiar phenomenon in laboratories utilizing experimentally the regenerating plumage. Forcible removal of the standing feather causes a tearing of the apical tissue of the papilla with haemorrhage into the lumen of the follicle(1). The wound stimulus is sufficient to assure immediate activation of the papilla in the capon whereas in the cock and hen, regeneration usually fails outside of the environs of molt. We may speak at such times of a "quiescent papilla." The papilla has long been shown to respond by

growth to oral or parenteral administration of thyroid substance(2; and many others). The dosage requisite for this reaction was less as the season of normal molt approached(3), showing the gradual course of natural changes which culminate in that event. Recently it has been established that progesterone, when given as a single intramuscular injection of a preparation having slow absorption from local depots (Reposito Progesterone), also will cause a general activation of the feather papillae in adult fowl(4). There was no hint in these experiments that progesterone action was here in any sense *via* a stimulation of the bird's thyroid; this was checked in the feather structure where excess thyroid has long been known to produce specific, readily recognized consequences(5; and many others). The similar end effects seem accordingly independently due to either progesterone or to thyroxine although some common intermediate participation is not ruled out.

This possibility suggested following the consequences of separate local applications of either of the two substances to the papilla;

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these observations are recorded here.

Methods. Adult hens bearing feather papillae in the various physiological states were used. The test substance was applied to an area of one side of the bird's body and as control the respective solvent was applied to an identically prepared site in the opposite location. Progesterone was administered as a single intradermal injection of 0.20 ml of a solution of 50 mg/ml in sesame oil or as a daily local application during 4 weeks of progesterone in ointment, 50 mg/g.[†] Thyroxine was prepared as a solution of 5 mg (Thyroxine crystals Squibb) in 1 ml distilled water plus 1 drop of a 4% sodium hydroxide solution. It was administered as a single intradermal injection of 0.2 ml or as a daily repeated local application in ointment during 4 weeks. One-half ml of the thyroxine solution was rubbed up in a small amount of a commercial vanishing-type cream, the procedure repeated as required. Progesterone in oil was also injected directly into the superior umbilicus of the large secondaries in the expectation that the cavity of the quill of the standing feather would provide a source of gradual supply to the underlying papilla. This proved negative. Previous experiments had shown thyroxine in the same situation to enter into the general circulation and this route to be unsuited to the purpose.

Results. Five birds were treated with progesterone in ointment and 5 with thyroxine in ointment; in 3 of each series, the application was made to simultaneously defeathered areas; in the other 2, application was to the skin spaces separating adjacent standing feather rows. No activation was elicited in the papilla in either circumstance. Thyroxine in some amounts, however, apparently penetrated the epidermal tissues as there were subsequently noted characteristic changes of pigmentation in regenerating feathers of distant location. Thyroxine was given by intradermal injection on 28 occasions, progesterone on 27 occasions. The birds used were prepared so as to afford the 3 classes of papillae to either

TABLE I. Local Action on Feather Papilla of Thyroxine and of Progesterone.

Treatment	Papilla state	No. trials	Feather germs regenerated (No.)	
			Treated side	Control side
Thyroxine (i.d.)*	Defeathered	15	119	59
	Resting	10	33	0
	Quiescent	3	10	0
Progesterone (i.d.)	Defeathered	15	96	58
	Resting	8	0	0
	Quiescent	4	9	10

* Intradermal.

test substance. Birds were defeathered in small areas to assure the additional stimulus of plucking; the immediately following injections were made between adjacent denuded follicles. In affecting the resting papilla, the site injected was between 2 rows of standing feathers. The birds considered to have quiescent papillae had been plucked about 2 months earlier and had not exhibited regeneration up to the date of this treatment. The data from these series are summarized in Table I. The compilations refer to the total number of feather germs which were regenerated in the immediate vicinity of the point of injection. Examination of Table I shows thyroxine in intradermal injection to effect local proliferation of all types of the papilla. Experimental activation in resting and quiescent states, where it is opposed to zero regeneration in the control side, could not be clearer. However, the augmentation of the stimulus brought to growth in preliminary defeathering is equally evident. The properties of progesterone in these respects are less distinct. As administered, progesterone was negative in the resting papilla and the records of the quiescent papilla must be discounted in view of the regeneration occurring in the control area. In defeathering, a greater number of regenerations occurred in progesterone treatment which may suggest an increasing importance as the seasonal inertia of the papilla lessens. It is evident that the papilla has an annual or biannual physiological cycle which may proceed at very different levels (*cf.* capon and sexes of fowl), and which is triggered to an external event, presumably some hormonal impulse or withdrawal. If

[†] Progesterone was supplied generously by the Research Department of Ciba Pharmaceutical Products, Summit, N. J.

further studies should definitely prove progesterone to be locally negative in the feather papilla, this would show its systemic action to be upon some intermediate link. Since the bird's own thyroid is apparently unaffected by progesterone in circumstances where the substance is adequate for growth induction in the papilla, that gland is probably not the participant despite the very evident experimental effectiveness. In these aspects, the newly discovered role of progesterone casts some doubt on the concept that surges of thyroidal activity initiate the normal events of molt and suggests a revised approach to an important problem.

Summary. The respective local actions upon the feather papilla of thyroxine and of progesterone were examined. Application was

made by intradermal injection to the immediate vicinity of the resting, quiescent and plucking-stimulated papillae. Thyroxine was effective in causing proliferation in all 3 circumstances; progesterone proved negative with this technic in the resting and quiescent states; it may have exerted a slight augmentation in the third.

1. Lillie, F. R., and Juhn, M., *Physiol. Zool.*, 1932, v5, 124.

2. Larionov, W. Th., and Kusmina, N., *Biol. Zentralblatt*, 1931, v51, 81.

3. Van der Meulen, J. B., *Proc. 7th World's Poultry Congress*, 1939, p109.

4. Shaffner, C. S., *Science*, 1954, v120, 345.

5. Cole, L. J., and Reid, D. H., *J. Agr. Research*, 1924, v29, 285.

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Fate of Ascorbic Acid in Early Radiation Damage.* (21983)

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(Introduced by F. Schlenk.)

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There is a paucity of information regarding the role of ascorbic acid in radiation damage. In light of the reported involvement of ascorbic acid in the conversion of folic acid to citrovorum factor(1) and, thereby, in nucleic acid synthesis, its sensitivity *in vivo* to irradiation is of interest. Thus far, the study of ascorbic acid has been concerned chiefly with its changes in the adrenal gland(2). We have investigated a variety of rat tissues from the standpoint of the concentration and total organ content of ascorbic acid as well as organ weight after whole-body X-irradiation. These organs included cervical lymph nodes, thymus, liver, spleen, kidney, intestinal mucosa, testis, brown adipose tissue, muscle, adrenals, and brain. The results indicate a highly selective action of irradiation on tissue ascorbic acid.

Methods. Male Sprague-Dawley rats, weighing 125-200 g, receiving a stock Purina

Chow diet *ad lib.*, were irradiated at a rate of 23 r/min with a 250-kvp X-ray machine at 15 ma with a 0.5 mm Cu plus a 1.0 mm Al filter and the HVL equal to 2.0 mm of Cu. The target distance was 34 inches from the table supporting the animals. At 4, 28, and 72 hours after irradiation, groups of 4 to 6 animals were sacrificed. The organs were dissected out, blotted, and weighed on a torsion balance. A 9-inch strip of small intestine was slit, washed, and scraped to provide a sample of intestinal mucosa. The tissues were homogenized in 10% trichloroacetic acid; all of the ascorbic acid was converted to the oxidized (dehydro) form and determined as the osazone by the method of Roe and Kuether (3). No differentiation of oxidized and reduced ascorbic acid was carried out. Typical control values and their variation are shown in Table I. The values for concentration, total ascorbic acid and organ weight for the experimental groups are reported as a percent of the control values.

* Work performed under the auspices of the U.S. Atomic Energy Commission.

TABLE I. Ascorbic Acid Concentration and Organ Weights of Normal and Chloretone-Treated Rats.*

Organ	Normal		Chloretone-treated†	
	Ascorbic acid, mg %	Organ wt, mg/g	Ascorbic acid, mg %	Organ wt, mg/g
Adrenals	311.6 ± 28.8	.21 ± .01	391.4 ± 16.9	.20 ± .00
Thymus	66.8 ± 3.2	4.02 ± .57	64.4 ± 2.3	2.92 ± .29
Spleen	49.7 ± 2.9	4.44 ± .16	51.5 ± 2.2	3.56 ± .39
Cervical lymph nodes	46.5 ± 2.0	.81 ± .06	60.5 ± 3.0	.93 ± .17
Brain	41.7 ± .2	9.18 ± .32	38.6 ± .9	8.13 ± .04
Intestinal mucosa, 9 in.	33.0 ± 2.7	2.03 ± .04	38.0 ± 3.1	1.84 ± .12
Left testis	29.9 ± .9	5.70 ± .16	28.5 ± 1.3	5.72 ± .63
Liver	27.2 ± .9	54.5 ± 4.0	36.7 ± 1.3	61.3 ± .2
Left kidney	16.0 ± .1	4.87 ± .17	19.3 ± 1.4	5.34 ± .24
Pancreas	6.5 ± .5	3.62 ± .46	6.7 ± .5	2.50 ± .04
Skeletal muscle	5.0 ± .3	—	4.6 ± .3	—
Brown adipose tissue	4.5 ± .4	1.68 ± .12	4.7 ± .5	1.18 ± .12

* Avg of 3 animals and its stand. dev.

† 1 mg of chloretone/ml drinking water for 6 days.

Results. After exposure to 600 r there were no gross changes in concentration or organ content of ascorbic acid or in organ weight in liver, brain, pancreas, testis (Fig. 1), muscle, kidney and brown adipose tissue. The adrenal glands showed an immediate drop in concentration at 4 hours, as has been noted by other workers(2); however at 28 hours the level had returned to normal and remained unchanged thereafter. The remaining 4 tissues: thymus, lymph nodes, spleen, and intestinal mucosa, underwent the rapid changes shown in Fig. 2. The total ascorbic acid content of lymph nodes, thymus, and spleen decreased to 30, 25, and 10%, respectively, of the original values. The concentration of ascorbic acid in these organs dropped abruptly within 4 hours to 65-70% and changed slowly thereafter. After 300 r, the changes in these organs were similar to those observed at the higher dose level while after 100 r the losses of ascorbic acid were small from the thymus and spleen and negligible from the lymph nodes.

The changes observed in the intestinal mucosa 4 hours after X-irradiation were unique. Despite a loss of tissue weight, the total ascorbic acid content of this tissue had increased because of the concentration which had risen to 170% of the control value. This response likewise diminished slightly with lower doses of irradiation, but, even at 100 r, the concentration of ascorbic acid was 135% of the control value 4 hours after irradiation.

We investigated the water content of these four tissues after irradiation to determine, in the thymus, lymph nodes, and spleen, if there is an edema present which masks a loss of cells containing high concentrations of ascorbic acid, or in the mucosa, if there has been a dehydration of the tissue which caused an apparent increase in concentration. From Table II (which shows percent water content of organs of rats after exposure to 600 r), it can be seen that changes in water content of tissues could not account for the observed changes in ascorbic acid concentrations. In the lymph nodes there was even some dehydration.

The effect of stimulating synthesis of ascorbic acid on subsequent response to irradiation was next investigated. Administration of chloretone at a level of 20 mg/day for 7 days has been shown to result in urinary excretion of as much as 20-30 mg of ascorbic acid per day, in contrast to normal excretion of 0.5-2.0 mg/day(4). It does not necessarily follow, however, that concentration of ascorbic acid in tissues is correspondingly increased, since a low kidney threshold has been shown to exist in other species(5). That this appears to be the case may be seen by comparing the values for ascorbic acid concentration of tissues of rats given chloretone (1 mg/ml of drinking water) with those of untreated animals (Table I). Tissues of rats treated with chloretone exhibited, for example, the following increases in ascorbic acid concentration: liver, 35%;

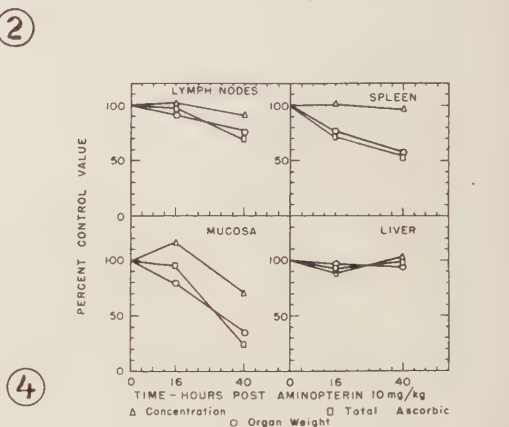
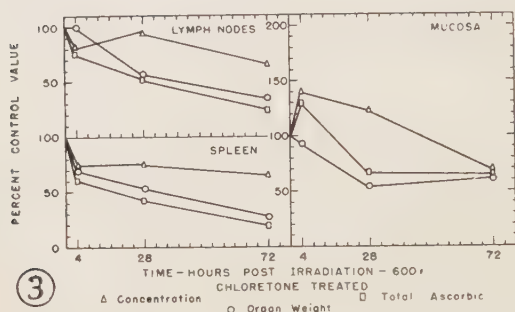
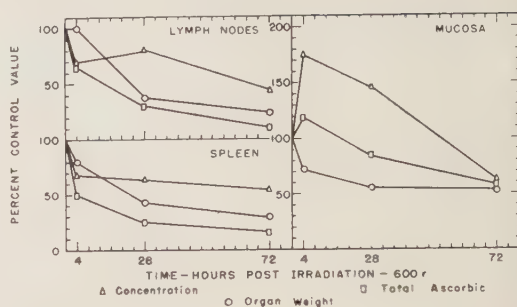
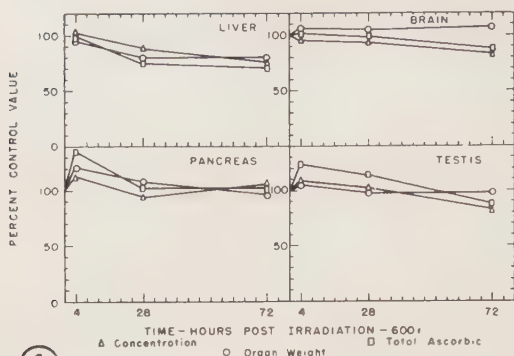


FIG. 1. Ascorbic acid changes in normal rat liver, brain, pancreas, and testis after 600 r.

FIG. 2. Changes in ascorbic acid in normal rat lymph nodes, spleen, and intestinal mucosa after 600 r.

FIG. 3. Ascorbic acid changes in chlorotone-treated rat lymph nodes, spleen, and intestinal mucosa after 600 r.

FIG. 4. Changes in ascorbic acid in rat lymph nodes, spleen, intestinal mucosa, and liver after aminopterin treatment.

lymph nodes, 30%; intestinal mucosa, 15%; and spleen, 4%. These animals served as chlorotone-treated, non-irradiated controls for the following irradiated animals. The remaining 24 rats were irradiated with 600 r and chlorotone administration continued after irradiation. Their tissues displayed changes in concentration of ascorbic acid, total content and organ weight (Fig. 3) similar to those seen in previous experiments. The effect of chlorotone was apparent in higher final levels of ascorbic acid in tissues of treated animals.

and in a slight reduction of the rise in mucosal concentration. Administration of chlorotone did not, however, abolish the decrease in concentration in lymphoid organs or stimulate recovery within 72 hours.

Alternatively, the effect of blocking the conversion of folic acid to citrovorum factor on the levels of ascorbic acid was studied. When aminopterin was administered intraperitoneally in doses of 10 mg/kg of body weight to a group of rats, the concentration and total content of ascorbic acid in lymph nodes, spleen, mucosa, and liver underwent the changes shown in Fig. 4. Aminopterin caused an involution of lymph nodes and spleen, but the loss of total ascorbic acid paralleled the loss in tissue weight, leaving the concentration unchanged. There was a small but significant rise in mucosal concentration after 16

TABLE II. Organ Water Content.

Hr post-irradiation	% organ wt		
	Lymph	Spleen	Mucosa
0	81.8	78.1	81.7
4	76.9	76.0	80.7
28	79.5	77.3	82.0
72	77.8	75.5	82.8

hours, and no changes were observed in the liver.

Discussion. After irradiation with 600 r the majority of organs analysed presented no striking changes in ascorbic acid within 72 hours. However, organs of a lymphoid nature quickly dropped in both concentration and total content of ascorbic acid, while the mucosa increased in concentration during the same interval. Certain possibilities appear evident as explanations of the phenomena. For example, it is tempting to explain the rise in mucosal concentration and decreases in lymphoid tissue by means of a migration of lymphocytes to the intestinal epithelium. The rise in mucosal concentration after aminopterin treatment lends credence to this possibility, since such a migration has been observed after both aminopterin treatment and X-irradiation(6,7) and a histological examination of the small intestine confirms the presence of increased number of lymphocytes.[†]

The influx of lymphocytes cannot be wholly responsible for the findings because the increase in lymphocytes appeared to be insufficient to account for the total rise and the accumulation of such cells was not, apparently, matched by a corresponding loss of material from the lymphoid organs themselves.

The stability of ascorbic acid concentration in these organs after treatment with aminopterin would suggest that blocking conversion of folic acid to citrovorum factor does not in itself affect concentration of ascorbic acid. These data are, themselves, at variance with those of Williams(8) and Schwartz and Williams(9) who found a decrease in liver ascorbic acid concentration 13 days after feeding of aminopterin at a level of 4 mg/kilo of diet. This discrepancy may be due either to route of administration or to high dosage (LD₁₀₀—72 hours) employed in the present study which was, in our consideration, necessary to duplicate the acute aspect of irradiation. Even at this level, however, the concentration

of ascorbic acid in tissues did not decrease within 40 hours.

Furthermore, the effect of stimulating the synthesis of ascorbic acid on changes in concentration or organ content of ascorbic acid after irradiation is negligible, suggesting that an impairment of the synthetic process does not seem to be responsible. Nor does the stimulation of ascorbic acid synthesis by chloretone seem to be abolished by irradiation.

This then focuses attention on the relationship of ascorbic acid to the particular tissue in which its concentration is so readily changed by X-ray. The observed rapid rise of ascorbic acid and the well known inhibition of mitosis in the intestinal mucosa after irradiation suggest that the accumulation of ascorbic acid is a result of arrested mitosis and that ascorbic acid is requisite to and/or consumed by mitotic activity. A similar suggestion has been made by Stern and Timonen (10) working with lily anthers. Failure to observe similar changes in the other tissues may be due to their relatively much lower mitotic activity. Coupled with this, the loss of ascorbic acid from lymphoid tissues may be explained by the known radiation-induced fragility of several cell types in relatively high concentration in these organs.

Summary. 1. There were no gross changes in concentration or total content of ascorbic acid within 72 hours after 600 r of X-ray in rat liver, brain, pancreas, testis, muscle, kidney, or brown adipose tissue. Thymus, lymph nodes, and spleen showed a drop in ascorbic acid concentration within four hours, and the content of ascorbic acid continued to drop throughout the period studied. Intestinal mucosa showed a sharp increase in ascorbic acid concentration during the first 4 hours followed by a gradual decline. 2. Stimulation of ascorbic acid synthesis by chloretone administration does not modify the effects of irradiation, nor does the hydration of these tissues change in a fashion which might account for the observed phenomena. 3. The observed changes in concentration could not be duplicated by the administration of aminopterin at a level

[†] We wish to thank Dr. F. Wassermann for preparation of the material and Dr. A. M. Brues and Mrs. Agnes Stroud for assistance in interpretation of the specimens.

of 10 mg/kg body weight.

1. Nichol, C. A., and Welch, A. D., *PROC. SOC. EXP. BIOL. AND MED.*, 1950, v74, 52.
2. Oster, H. L., Kretchmar, A. L., and Bethell, F. H., *ibid.*, 1953, v84, 470.
3. Roe, J. H., and Kuether, C. A., *J. Biol. Chem.*, 1943, v147, 399.
4. Jackel, S. S., Mosbach, E. N., Burns, J. J., and King, C. G., *ibid.*, 1950, v186, 569.
5. Friedman, G. J., Sherry, S., and Ralli, E. P., *J. Clin. Invest.*, 1940, v19, 685.

6. Phillips, F. S., Thiersch, J. B., and Ferguson, F. C., *Ann. N. Y. Acad. Sci.*, 1950, v52, Art. 8, 1349.
7. Pierce, M., *Histopathology of Irradiation*, 1948, 502, W. A. Bloom, ed., McGraw-Hill, N. Y.
8. Williams, J. N., Jr., *PROC. SOC. EXP. BIOL. AND MED.*, 1951, v77, 315.
9. Schwartz, M. A., and Williams, J. N., Jr., *J. Biol. Chem.*, 1952, v194, 711.
10. Stern, H., and Timonen, S., *J. Gen. Physiol.*, 1954, v38, 41.

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Relationship of Prolonged Drainage of Bile through Pancreatic Duct System to Pancreatitis.* (21984)

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The role of reflux of bile into the pancreatic duct system in the etiology of acute hemorrhagic pancreatic necrosis has been a subject of intensive investigation for many years. It is well known that when sterile bile is injected into the pancreatic duct of experimental animals, under certain conditions, a fulminating hemorrhagic pancreatic necrosis results quite regularly. However, there is a great deal of evidence that under other more physiologic conditions the presence of bile in the pancreatic duct is harmless to the pancreas. Mann and Giordano(1) found that, if the pressure exerted during retrograde injection of bile into the pancreatic duct were maintained within physiologic limits, no pancreatitis developed. It was shown in this laboratory(2) in cats that when the common bile duct was occluded distal to the entrance of the pancreatic duct so that bile could flow into the pancreatic duct under secretory pressure of the liver or pressure of the contracting gall bladder, pancreatitis developed in only a small proportion of animals, unless the cats were fed fatty meals supplemented with bile

salts. Leven(3) and Colp and Doubilet(4) showed that reflux of iodized oil into the pancreatic duct could be demonstrated in cholangiograms in about 20% of patients with common bile duct drainage. This reflux was attributed in most cases to spasm of the sphincter of Oddi. Hicken and McAllister(5) using a water soluble contrast agent and very low injection pressures found that even in patients with no intraductal pathology, reflux of contrast material into the pancreatic duct could be demonstrated on routine cholangiograms in a high percentage of cases. Fisher *et al.*(6) drained the bile through the pancreatic duct by connecting a catheter in the common bile duct with a catheter in the pancreatic duct. In their 4 experiments the longest follow-up was 7 days. None of these animals developed any pancreatitis.

The experiments reported here, from August 1950 to July 1951, were designed to show the effect upon the pancreas of draining all of the bile secreted by the dog's liver through the pancreatic duct system over long periods of time.

Methods. The entire biliary flow in a series of dogs was caused to pass through the pancreatic duct into the duodenum by one of 3 operations. Type I: the duct of Santorini

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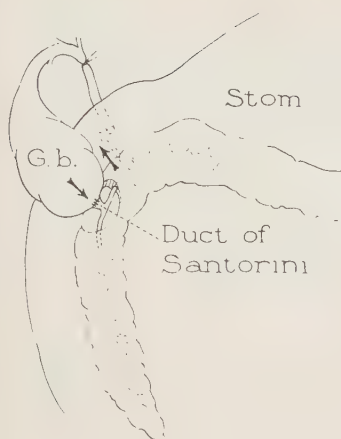


Fig. 1

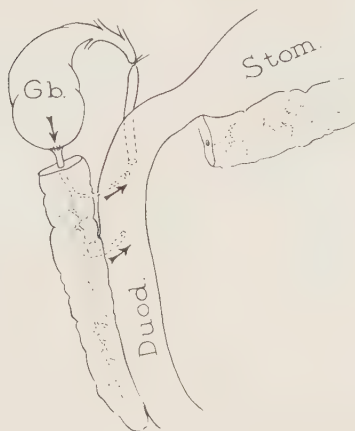


Fig. 2

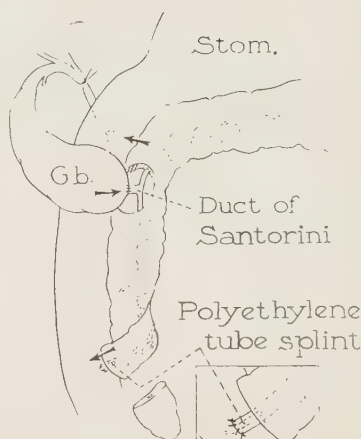


Fig. 3

FIG. 1, 2 and 3 show Type I, II and III operations, respectively. Bile drains through pancreatic duct system as indicated by arrows.

(the larger duct in the dog) was anastomosed to the gall bladder and the common bile duct was tied off. Under these circumstances, bile flows from the duct of Santorini into the duct of Wirsung through anastomoses between the two duct systems which are regularly present in the dog and thence into the intestine through the duct of Wirsung. Type II: the body of the pancreas was transected and the cut end of the pancreatic duct in this part of the pancreas was anastomosed to the gall bladder and the common bile duct was ligated. In this preparation bile flows from the gall bladder into the pancreatic duct through the anastomosis and reaches the intestine by way of the normal pancreatic duct orifices. Type III: the duct of Santorini was divided just proximal to its orifice in the duodenum, and this duct was anastomosed to the gall bladder. The distal end of the duodenal portion of the pancreas was transected and the cut end of the duct was located and anastomosed into the duodenum using a small polyethylene tube as a splint. The common bile duct was either ligated or anastomosed to the gall bladder. Bile flows into pancreas through the major duct and out into the intestine through the point where the duct is implanted into the duodenum. Serum amylase and serum bilirubin determinations were made on all dogs at weekly intervals.

Results. Of the 47 dogs used in these ex-

periments, 18 had patent functional anastomoses between the gall bladder and the pancreatic duct system and between the pancreatic duct and intestine at the time that they died or were sacrificed, and these are the animals included in this study. Eleven of the remaining animals are not included because at the time of death it was found that the anastomosis between gall bladder and pancreatic duct or between pancreatic duct and intestine was not patent, and 18 were not included either because they did not survive for a significant period after their operation or because there was insufficient data on them.

Of the 18 dogs with intact anastomoses, only 3 had clinically significant degrees of pancreatitis, and only one of these had a typical full-blown pancreatic necrosis. The case of severe pancreatic necrosis occurred 22 days postoperatively in a dog in which a normal serum bilirubin and only a moderately elevated serum amylase had been noted only 4 days earlier. The other 2 dogs had only local areas of necrosis in the pancreas.

Fifteen of the 18 dogs with intact anastomoses did not develop significant degrees of pancreatitis. Eight were not jaundiced at the time of death, indicating that the bile was passing relatively unimpeded through the pancreatic duct system into the intestine. Four of these 8 dogs lived from 58 to 138 days, and

the average survival time for the group was 60 days. Gross inspection at autopsy showed the pancreas to be firm and atrophic; the gall bladder and bile ducts were dilated and the liver was firm and rough. Microscopic examination of the pancreas usually showed only mild degrees of interstitial pancreatitis and fibrosis.

The remaining 7 dogs with intact anastomoses had a significant degree of clinical jaundice at the time of death even though a patent channel was demonstrated from the gall bladder through the pancreatic duct system into the duodenum at autopsy. This circumstance suggests that although some bile was probably passing through the pancreatic duct system, the flow was not free enough to maintain the serum bilirubin at normal levels.

Summary and conclusions. 1. The entire flow of bile was successfully diverted through the pancreatic duct system into the intestine in a total of 11 dogs. Eight of these dogs did not develop any significant degree of pancrea-

titis even though the average observation period for this group was more than 60 days, and only one dog in the series developed a full-blown acute pancreatic necrosis. 2. The results of these experiments indicate that the presence of bile in the pancreatic duct system under physiologic pressures is tolerated for long periods of time without any serious damage to the pancreas in most cases.

1. Mann, F. C., and Giordano, A. S., *Arch. Surg.*, 1923, v6, 1.
2. Wangenstein, O. H., Leven, N. L., and Manson, M. N., *Arch. Surg.*, 1931, v23, 47.
3. Leven, N. L., *PROC. SOC. EXP. BIOL. AND MED.*, 1938, v38, 808.
4. Colp, R., and Doubilet, H., *Surgery*, 1938, v4, 837.
5. Hicken, W. F., and McAllister, A. J., *Am. J. Surg.*, 1952, v83, 781.
6. Fisher, B., Fisher, E. R., and Selker, R., *Surgical Forum Clinical Congress, Am. Coll. of Surg.*, 1953, p406, Saunders, Philadelphia, 1954.

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Lactic Dehydrogenase Activity in Blood.* (21985)

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The observation that during experimental and clinical myocardial infarction glutamic oxaloacetic transaminase is released from cardiac muscle resulting in increased enzyme activity in the serum(1-5), suggested that other cardiac tissue enzymes behave similarly during myocardial infarction. Although present in other tissues in greater activity, lactic dehydrogenase, the enzyme concerned primarily with the reduction of pyruvic acid to lactic acid, is present in appreciable activity in cardiac musculature. In order to ascertain whether lactic dehydrogenase (hereafter referred to as LD) activity is increased in the serum during myocardial infarction, it was necessary to first demonstrate its presence

in human and animal blood, and to delineate variations in LD activity in the blood of normal and diseased man.

Methods and materials. Lactic dehydrogenase is concerned with the reduction, in the presence of reduced diphosphonucleotide (DPNH), of alpha-keto and of alpha, gamma-diketo acids; maximum reduction by LD has been shown to occur with pyruvic acid. Inasmuch as LD catalyzes the reduction of various alpha-keto and alpha, gamma-diketo acids, the reduction of a substrate in the presence of LD cannot be used to specifically identify an alpha-keto or an alpha, gamma-diketo acid. However, the catalytic reduction of a known amount of a specific keto acid may be used to quantitatively estimate LD activity(6). The LD activity of human serum was measured

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spectrophotometrically. To 2.4 ml of pH 7.4 phosphate buffer, 0.1 m of serum and 0.1 m of reduced diphosphonucleotide (DPNH) (2.5 mg per ml) were added. After 20 minutes, 0.1 ml of sodium pyruvate (2.5 mg per ml) was added. The optical density at wavelength 340 $m\mu$ was followed for 3 to 5 minutes at intervals of 10, 15, or 30 seconds depending on the rate of reaction. The rate of decrease of optical density representing the rate of oxidation of DPNH was taken as the measure of LD activity of serum. The reaction was followed in a Beckman model DU spectrophotometer using a tungsten light source and at room temperature, 24 to 27°C. The activity is expressed as units per ml of serum per minute. One unit equals a decrease in optical density of 0.001 per minute per ml under the conditions described.

When DPNH and pH 7.4 phosphate buffer were added to serum without the addition of pyruvate, the optical density of the mixture decreased slowly for up to 20 minutes indicating the oxidation of a finite quantity of DPNH. This initial oxidation of DPNH by serum without added pyruvate appears to depend on the presence of alpha-keto and alpha, gamma-diketo acid in the serum (in the presence of serum LD). The reaction stopped when the keto and diketo acids were enzymatically reduced to lactate by DPNH. The addition of more DPNH will not restart the reaction, but the addition of pyruvate resulted in a steady decrease of optical density which was taken as a measure of LD activity of the serum. The optimal concentration of DPNH was found to be 0.1 ml (2.5 mg DPNH per ml in water made basic to pH 9.0 with sodium hydroxide) in a total reaction mixture of 3.0 ml (3.8×10^{-7} M). At pH 7.2 to 7.6, serum LD was optimally active and this is in substantial agreement with that reported for LD from other tissue sources(6-8). The LD activity of serum was maximal when 0.1 ml of pyruvate (2.5 mg per ml of distilled water) was used in a total reaction mixture of 3.0 ml (0.23×10^{-5} M). The rate of LD activity was directly proportional to the quantity of serum used. No activators of LD were used although it has been reported(9) that amino-DPNH potentiates LD activity. Competitive inhibitions of LD by alpha, gamma-

TABLE I. Distribution of Lactic Dehydrogenase Activity in Venous Serum of Normal Adults.

LD activity (units)	No. of individuals	%
250-290	7	4.3
300-340	18	11.2
350-390	25	15.5
400-440	32	20.0
450-490	22	13.7
500-540	16	10.0
550-590	17	10.6
600-640	7	4.3
650-690	7	4.3
700-740	2	1.2
750-790	4	2.5
800-840	3	1.9
850-890	1	0.7
Total	161	100.2

Mean LD activity, 470 units \pm 130 units.

diketo valerie acid has been reported(6) but the enzyme is insensitive to sulphydryl reagents(10). Serum samples stored from 30 minutes to 96 hours at room temperature or for periods of from 1 hour to 1 week in the refrigerator (0° to 5°C) showed no significant alteration in serum LD activity; the heat stability noted is in essential agreements with reports of LD derived from other sources(7, 11).

Results. 1. *Lactic dehydrogenase activity in blood* of human adults. The venous serum LD activity in 161 normal human adults varied from 260 to 850 units with a mean activity of 470 per m \pm 130 units (Table I). The LD activity found in whole venous blood hemolysates from normal human adults ranged from 16,000 to 67,000 units with a mean value of $34,000 \pm 12,000$ units per ml. The whole blood hemolysates were noted to be on the average approximately 100 times

TABLE II. Lactic Dehydrogenase Activity of Heparinized Plasma, Serum, and Whole Blood Hemolysate of the Same Venous Blood Samples.

Hematocrit (mm)	Whole blood hemolysate (units/ml)	Plasma (units/ml)	Serum (units/ml)
	$\times 1000$		
42	22	370	380
43	26	370	380
45	40	800	750
50	33	300	400
50	24	500	420
50	31	330	290
51	16	370	390
52	30	480	430
53	27	830	340
54	30	500	360

TABLE III. Estimated Lactic Dehydrogenase Activity of Tissues of the Dog.

Tissue	Units/g wet tissue ($\times 1000$)
Kidney	640
Skeletal muscle	600
Liver	390
Heart	240
Pancreas	150
Spleen	140
Brain	130
Lung	25

more active than the serum of the corresponding venous blood samples, but no consistent relationship was noted between serum and corresponding whole blood hemolysate LD activities, and none between whole blood hemolysate activity and hematocrit (Table II). The LD activity of plasma obtained from heparinized venous blood samples was not significantly different from the activity of serum from a corresponding venous blood sample, oxalated plasma was considerably less active suggesting oxalate inhibition and/or interference. In no instance was LD activity absent in the sera of normal human adults tested or in any of the sera of hospitalized patients with various disease states. The fasting did not appear to significantly influence the LD activity, and no consistent alteration in LD activity was observed during glucose tolerance studies in normal or in diabetic individuals. Day to day serum LD activities in the same individual varied 30% (within the normal range). Duplicate determinations on the same serum samples varied by 10% or less. LD activity was not connected with sex.

A sampling was made of patients hospitalized at Memorial Center with various diseases and serum LD activity determined in each instance. Activities within the normal range were obtained on sera from a limited selection of patients with infectious, degenerative, neoplastic and other disease states. High activities were observed in patients with myocardial infarction, diabetic acidosis, acute stem cell leukemia, chronic myelogenous leukemia and hepatitis.

2. *Lactic dehydrogenase activity in the tissues and blood of experimental animal.* LD activity was demonstrable in all the serum samples obtained from several experimental

animals. LD activity in the venous sera of the experimental animals studied varied from 380 units per ml in the rabbit to 3100 units per ml in the rat with progressively diminishing values in between these 2 extremes in the dog, hamster, mouse and guinea pig. Using aliquot samples of macerated homogenized dog tissues obtained immediately after the death of the animal, the LD activity of various tissues was estimated and are summarized in Table III.

Following the intravenous administration of 0.13 ml of lactic dehydrogenase[†] diluted with saline to 5.0 ml (equivalent to approximately 240,000 units of LD activity or about one gram of dog heart muscle homogenate) to a 12.5 kilo dog, the serum was sampled at periodic intervals and examined for LD activity as indicated in Fig. 1.

Fig. 2 describes the serum LD alterations following experimental myocardial infarction, produced by the closed chest technic (5). (The detailed observations of serum and cardiac tissue LD activity elevations seen in experimental graded myocardial infarction and in clinical myocardial infarction will be the subject of a subsequent report.) As seen in the case of glutamic oxaloacetic transaminase, serum LD rises during the course of myocardial infarction. Similar alterations in serum LD activity have been observed in clinical myocardial infarction (Fig. 3).

Discussion. Lactic dehydrogenase activity

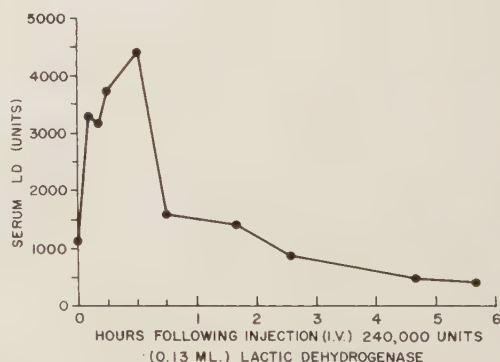


FIG. 1. Serum lactic dehydrogenase activity in a 12.5 kilo dog following the intravenous injection of approximately 240,000 units of lactic dehydrogenase.

[†] Obtained from Nutritional Biochemical Corp., Cleveland, O.

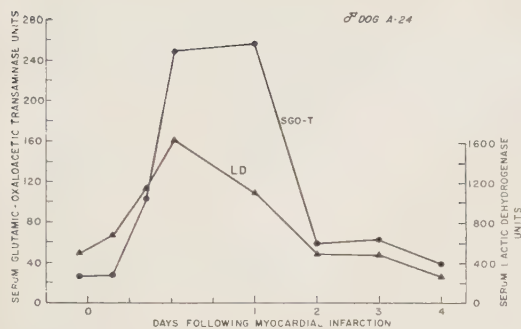


FIG. 2. Serum lactic dehydrogenase alterations during the course of closed chest experimental myocardial infarction in the dog. Comparison with serial changes in serum glutamic oxaloacetic transaminase is shown.

is present in all human sera and in all whole blood hemolysates examined. The previously reported serum glutamic oxaloacetic transaminase activity alterations in the course of myocardial infarction appear to be mimicked by similar changes in LD activity. That the latter changes are parallel but not related to serum glutamic oxaloacetic transaminase activity alterations is indicated by a comparison of serum glutamic oxaloacetic transaminase and LD activity observed during the course of clinical hepatitis. The LD and serum glutamic-oxaloacetic transaminase activity alterations following myocardial infarction are comparable in direction and degree but probably independent of one another. The fact that elevation of both LD and serum glutamic oxaloacetic transaminase follow heart muscle injury suggests that these enzymes are liberated from the damaged muscle cells into the blood stream. If such is the case for these

two enzymes, there is every reason to expect that other cardiac tissue enzymes, when present in sufficient concentrations in the heart muscle, would also be released following damage to the heart muscle cells with a consequent change in the serum enzyme activity. This generalization is presently under investigation.

Summary and conclusions. 1. Lactic dehydrogenase activity is present in the venous serum of normal human adults. Normal activity ranges from 260 to 850 units per ml with a mean value of 470 ± 130 units per ml. 2. Venous whole blood hemolysates of normal adults have a lactic dehydrogenase activity varying between 16,000 to 67,000 units per ml with a mean value of $34,000 \pm 12,000$ units per ml. 3. Alterations in serum lactic dehydrogenase have been studied in a selected group of disease states. 4. Experimental and clinical myocardial infarction are associated with a rise in serum lactic dehydrogenase activity. 5. Lactic dehydrogenase like serum glutamic oxaloacetic transaminase rises in a characteristic fashion following myocardial infarction.

The authors wish to acknowledge the technical assistance of Martin Podgainy. Dr. P. Rueggesser participated in the study of experimental myocardial infarction in dogs.

1. LaDue, J. S., Wróblewski, F., and Karmen, A., *Science*, 1954, v120, 3117.
2. Karmen, A., Wróblewski, F., and LaDue, J. S., *J. Clin. Invest.*, 1955, v34, 126.
3. Agress, C. M., Jacobs, H. I., Glassner, H. F., Lederer, M. A., Clark, W. G., Wróblewski, F., Karmen, A., and LaDue, J. S., *Circulation*, 1955, v11, 711.
4. La Due, J. S., and Wróblewski, F., *ibid.*, 1955, v11, 871.
5. Nydick, I., Wróblewski, F., and LaDue, J. S., *Circulation*, 1955, v12, 161.
6. Meister, A., *J. Biol. Chem.*, 1950, v184, 117.
7. Straub, F. B., *Biochem. J.*, 1939, v33, 787.
8. ———, *ibid.*, 1940, v34, 483.
9. Pullman, M. E., Colowick, S. P., and Kaplan, N. O., *J. Biol. Chem.*, 1952, v194, 593.
10. Negelein, E., and Brömel, H., *Biochem. Z.*, 1939, v303, 231.
11. Kubowitz, F., and Ott, Paul, *ibid.*, 1943, v314, 94.

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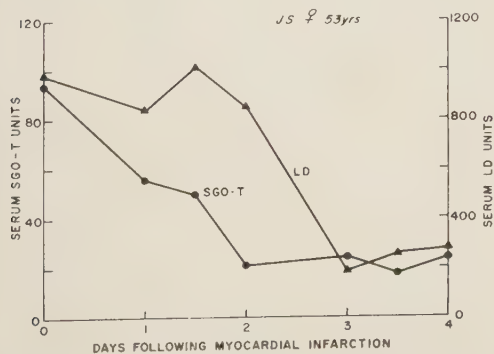


FIG. 3. Serum lactic dehydrogenase alterations during the course of clinical anterior myocardial infarction. Comparison with serial changes in serum glutamic oxaloacetic transaminase is shown.

Cytotoxic Action of Influenza Virus. Failure to Induce Acquired Resistance Phenomenon in Tissue Culture.* (21986)

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A number of substances modify the toxic action of influenza and related viruses in the intact animal. Sublethal doses of homologous or heterologous virus injected 24 hours prior to the challenge toxic inoculum prevent fever and lymphopenia in rabbits(1), convulsions in mice(2), and hemorrhagic encephalitis in chick embryos(3). Pretreatment of animals with *Cholera vibrio* filtrates also inhibits the pyrogenic(4) and neurotoxic(2) action of influenza viruses. Xerosin, a microbial product derived from *Achromobacter xerosis*, has the most profound inhibitory effect on the toxic pneumonitis(5) and encephalitis(6) caused by influenza and Newcastle disease viruses.

Henle, Girardi, and Henle(7) recently demonstrated that 4 strains of influenza virus produce a non-transmissible cytopathogenic effect in tissue cultures of a human epidermoid carcinoma (strain HeLa, Gey). Replication of infectious virus particles could not be demonstrated in this system but an increase in cellular non-infectious hemagglutinins occurred similar to the formation of "incomplete" virus in mouse brain that accompanies the neurotoxic action of influenza virus(8). This *in vitro* response of HeLa cells afforded a convenient method for determining whether biological inhibitors of influenza virus toxicity acted by interfering directly with host cell-virus interaction.

Materials. HeLa cell culture tubes were obtained from Microbiological Associates Inc. and incubated for an additional 24 hours at 35°-36°C before replacing the growth medium. The cells were then washed 3 times with 1 ml volumes of Hanks' balanced salt solution and the medium replaced with 0.8 ml of Scherer's

maintenance solution and 0.2 ml of horse serum. After an additional 3 days of incubation a final change of medium was made to 10% horse serum-maintenance solution prior to inoculation of the cultures. Intact sheets of healthy cells usually persisted in control tubes for at least 3-4 days longer, the duration of the experiment. *Virus* preparations with high toxic activity were prepared by allantoic inoculation of 10-11-day-old chick embryos with a 10⁻⁶ dilution of seed material. After incubation for 48-72 hours at 35.5°C the allantoic fluids were harvested, pooled, tested for hemagglutinin (HA) content and stored at -20°C. It was not necessary to concentrate these preparations but since their cytotoxic action diminished on storage, fresh material was prepared frequently. To obtain virus preparations of lower toxicity, eggs were inoculated with undiluted seed(9) and incubated for 72 hours. Most experiments were carried out with an egg-passage line of the Lee strain of influenza B virus. Certain comparisons were made with the mouse-pathogenic WS strain and the mouse-unadapted A/England/1/51 strain of influenza A virus. *Cholera filtrate* was prepared from the 4Z strain of *V. cholerae* and used both as the crude filtrate and concentrated, purified material with receptor-destroying enzyme (RDE) activity (10) of 5,000 units/ml. *Xerosin* was kindly supplied by Dr. Vincent Groupé. It was

TABLE I. Cytotoxic Effect on HeLa Cell Cultures of Lee Virus Added to the Medium 24 Hr after Cholera Filtrate or Xerosin.

	Controls (NAF)	Lee virus
Distilled water	—	4, 4, 4, 4, 4
RDE, 10 units	0, 1, 0, 0, 0*	4, 4, 4, 4, 4
" , 100 "	0, 0, 0, 0, 0	4, 4, 4, 4, 4
Xerosin, 100 µg	0, 0, 0, 0, 0	2, 4, 4, 4, 4
" , 1000 "	4, ±, 0, 1, 0	4, 4, 4, 4, 3

* 0, no cytopathogenic effect in 48 hr. 4, all cells destroyed. ± to 3, intermediate stages of cellular destruction.

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TABLE II. Influence of Subtoxic Virus, Cholera Filtrate and Xerosin Added to HeLa Cell Cultures 1 Hr before a 60% Cytotoxic Dose of Lee Virus.

	Controls (NAF)	Lee virus
Distilled water	0, 0, 0, 0	3, 2, 4, 2, ± 1, 3, 1, 2, 4
Subtoxic Lee virus	0, 0, 0, 0	3, 2, 4, 4, 4
RDE, 100 units	0, 0, 0, 0, 0	2, 3, 1, 1, 1
Xerosin, 1000 µg	0, 0, 0, 0	3, 3, 3, 4, 3

Read on scale of 0-4+ cytopathogenicity 72 hr after challenge.

dissolved in distilled water to the desired concentration and sterilized by autoclaving.

Results. Inoculation of HeLa cell cultures with 0.1 ml of undiluted "toxic" Lee allantoic fluid produced characteristic cytopathogenic effects. In 24 hours the cells were usually maintained as an intact sheet but islands of rounded cells with granular cytoplasm could be seen. By 48 hours after virus inoculation a considerable amount of destruction and clumping of cells was noted and by 72 hours most of the cells had dropped off the glass and could be seen floating in the medium. A 10-fold dilution of the Lee virus inoculum resulted in delayed and incomplete cytopathogenic effects and with a 1:100 dilution most of the cultures were unaffected. Allantoic fluid preparations of Lee virus made from undiluted seed, but possessing the same hemagglutinating activity as the "toxic" virus, were approximately one-tenth as potent.

Various concentrations of "subtoxic" virus, cholera filtrate and xerosin were incorporated into the maintenance medium, allowed to react with the cells, and the cultures challenged one or 24 hours later with 0.1 ml of toxic Lee virus. "Positive" virus controls consisted of tubes incubated in horse serum-maintenance solution with equivalent amounts of distilled water; "negative" controls, containing the same concentrations of test materials, were "challenged" with 0.1 ml of normal allantoic fluid (NAF). Distilled water, 200 AD of subtoxic virus, cholera filtrate and NAF had no effect on the microscopic appearance of the HeLa cells even after prolonged incubation. Minimal, sporadic cellular changes were observed in tubes containing 1 mg of xerosin, but these could be readily differentiated from typi-

cal virus pathogenicity. Ordinarily 5 or more tubes were used for each concentration of test material and "blind" readings made every day for 4 days after virus challenge. The extent of cellular damage was expressed on a scale of 0 to 4+. The numbers of test cultures are not always equal because it was necessary to discard an occasional contaminated tube.

Table I shows that the presence in the media of high concentrations of cholera filtrate and xerosin for 24 hours prior to Lee virus challenge did not inhibit cytotoxicity. In another experiment, a series of HeLa cultures were exposed to 200 AD of sub-toxic virus, 100 RDE units of cholera filtrate or 1 mg of xerosin and challenged one hour later with a preparation of Lee virus that produced only a 60% cytopathogenic effect in 72 hours. Under these conditions, it was again noted that these materials were ineffective as anticytotoxic agents (Table II). Lastly, in order to afford the most favorable conditions for demonstrating potential inhibition of cytotoxic action, a dose of toxic Lee virus was used that was capable of producing only 1+ cytopathogenicity in 72 hours. Cell cultures incubated for 24 hours with subtoxic Lee virus and xerosin were challenged with this minimally effective concentration of Lee virus. As shown in Table III, not only was there no inhibition of cellular damage, but pretreatment of cells with subtoxic virus or as little as 1 µg of xerosin enhanced the cytotoxic action of Lee virus in the 72-hour cultures. Even washing the cells free of xerosin and replacing the maintenance solution prior to virus inoculation rendered the cells more susceptible to the cytotoxic effects.

Many of these experiments were repeated with the WS and A/England/1/51 strains of influenza A virus. Although these viruses had more limited cytopathogenic action than the Lee strain, it was again noted that homologous or heterologous subtoxic virus, 100 RDE units of cholera filtrate (purified and crude) and 100-1000 µg of xerosin were incapable of preventing their *in vitro* cytotoxicity. In contrast, all of these materials effectively counteracted the neurotoxic action of the 3 strains of influenza virus. Mice injected intracerebrally with 200 AD of virus, 50 RDE units of cholera filtrate, or 30 µg of xerosin were highly re-

TABLE III. Apparent Enhancement of Minimally Cytotoxic Lee Virus by Subtoxic Virus and Xerosin.

	Control readings		Lee virus readings	
	72 hr	96 hr	72 hr	96 hr
Distilled water	0, 0, 0, 0, 0	0, 0, 0, 0, 0	1, 1, 1, 1, 1	4, 3, 3, 4, 3
Subtoxic Lee virus	0, 0, 0, 0, 0	0, 0, 0, 0, 1	3, 1, 1, 3, 2	4, 4, 2, 4, 4
Xerosin, 1 μ g	0, 0, 0, 0	0, 0, 0, 0	4, 3, 4, 4, 4	4, 4, 4, 4, 4
" , 1 " (washed)*	—	—	3, 3, 4, 4	4, 4, 4, 4

* Cultures incubated with 1 μ g of xerosin for 24 hr and washed 3 times before virus challenge.

sistant to the convulsive and lethal effects of 5 LD₅₀ of influenza virus administered 24 hours later.

Discussion. In the original studies(1) on acquired resistance of the intact host to virus toxins, the evidence seemed to support a local alteration in cellular susceptibility as the mechanism of action. This conclusion was based in part on the known capacity of virus and cholera filtrate to destroy the cell membrane receptors for influenza viruses(10). However, it was subsequently demonstrated that the degree of tolerance to the fever-producing(11,12) and neurotoxic(2) effects of influenza and mumps virus was not dependent on the enzymic action or receptor-gradient position of the protective virus. In addition, it is now quite clear that the antitoxic property of cholera filtrate is unrelated to receptor-destroying enzyme. Wagner(2) intimated that partially purified RDE was less effective in producing resistance to virus neurotoxin than crude cholera filtrate and French(11) proved that highly purified, concentrated RDE had no antipyrogenic activity. Finally, Groupé and coworkers(13) conclusively ruled out RDE as the antineurotoxic principle by demonstrating that autoclaved cholera filtrate, devoid of receptor-destroying activity, was almost as effective as unheated cholera filtrate. In spite of these studies, the likelihood persisted that resistance to virus neurotoxicity was a local cellular phenomenon since the protective effects of both subtoxic virus and cholera filtrate were manifested only by intracerebral inoculation(2).

Unlike influenza virus and cholera filtrate, xerosin does not affect cellular virus receptors(5) and induces some tolerance to toxic pneumonitis(5) and encephalitis(6) when ad-

ministered parenterally subsequent to virus challenge. Nevertheless, it is most effective when injected intracerebrally 24 hours before influenza virus and the duration of tolerance is strikingly similar to that produced by subtoxic virus and cholera filtrate. For these reasons, Groupé and Hermann(6) postulated that xerosin might have two modes of action, one directly on host cell-virus interaction and the other indirectly on the host response. However, studies on xerosin inhibition of toxic pneumonitis led Ginsberg(14) to conclude that its influence was exerted purely by bolstering host resistance. He found that xerosin was effective by the subcutaneous route, and that it did not suppress the formation of "incomplete" virus nor prevent virus destruction of bronchiolar epithelium. Lastly, Ginsberg emphasized the nonspecific nature of xerosin-induced resistance by showing that it protected mice again non-viral, chemical pneumonitis.

The present studies demonstrated that the cytotoxic effects of influenza virus on HeLa cell cultures were not diminished by doses of subtoxic virus, cholera filtrate or xerosin in excess of those required to protect mice against viral neurotoxic action. The tissue culture methods assured direct exposure of susceptible cells to high concentrations of presumed antitoxic substances. If it can be assumed that the neurotoxic and cytopathogenic actions of influenza virus are related phenomena, it would appear that tolerance of the intact host is not due to local alteration in cellular susceptibility. It is apparent that the whole question of the mechanisms of acquired resistance to virus toxic action requires reexamination.

Summary. Subtoxic virus, cholera filtrate

and xerosin failed to protect HeLa cell cultures against the *in vitro* cytopathogenic effects of the Lee strain of influenza B virus and the WS and A/England/1/51 strains of influenza A virus. Smaller doses of these materials effectively rendered mice tolerant to the neurotoxic action of influenza viruses. The possible implications of these studies on the mechanisms by which the intact host acquires resistance to virus toxic action are discussed.

1. Bennett, I. L., Jr., Wagner, R. R., and LeQuire, V. S., *J. Exp. Med.*, 1949, v90, 335.
2. Wagner, R. R., *Brit. J. Exp. Path.*, 1952, v33, 157.
3. Burnet, F. M., and Fraser, K. B., *Aust. J. Exp. Biol. M. Sc.*, 1952, v30, 447.
4. Wagner, R. R., and Bennett, I. L., Jr., *J. Exp. Med.*, 1950, v91, 135.

5. Groupé, V., Pugh, L. H., and Levine, A. S., *PROC. SOC. EXP. BIOL. AND MED.*, 1952, v80, 710.
6. Groupé, V., and Hermann, E. C., Jr., *J. Immunol.*, 1955, v74, 249.
7. Henle, G., Girardi, A., and Henle, W., *J. Exp. Med.*, 1955, v101, 25.
8. Schlesinger, R. W., *PROC. SOC. EXP. BIOL. AND MED.*, 1950, v74, 541.
9. McKee, A. P., *J. Immunol.*, 1951, v66, 151.
10. Burnet, F. M., and Stone, J. D., *Aust. J. Exp. Biol. M. Sc.*, 1947, v25, 227.
11. French, E. L., *ibid.*, 1952, v30, 479.
12. Wagner, R. R., *PROC. SOC. EXP. BIOL. AND MED.*, 1953, v83, 612.
13. Groupé, V., Hermann, E. C., Jr., and Daugherty, R. M., *PROC. SOC. EXP. BIOL. AND MED.*, 1954, v87, 636.
14. Ginsberg, H. S., *Fed. Proc.*, 1954, v13, 494.

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Effect of Epinephrine Upon Level of Plasma Amino Acids in the Eviscerate Rat. (21987)

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Some hormones affect the level of plasma amino acids in the eviscerate animal. The effect of epinephrine upon the level of plasma amino acids has not been studied in the eviscerate preparation although it has an effect in the intact animal(1). In the present study of eviscerate rats, doses of epinephrine which had striking effects upon glucose tolerance did not affect the level of plasma amino acids in either the presence or absence of insulin until large doses of epinephrine were administered with glucose and insulin for 18 hours.

Methods. Male rats of the Sprague-Dawley strain were fed Rockland rat diet. At a weight of 250 ± 5 g non-fasted rats were anesthetized with cyclopal sodium and functionally eviscerated. The intestinal tract was removed but the liver remained *in situ* after its arterial and portal vessels were tied. Continuous intravenous infusions of solutions of glucose with and without insulin and with and without epinephrine (Parke-Davis Adrenalin Chloride) were made by a continuous injection

machine. The fluid was delivered into the saphenous vein of the right hind leg at the rate of 20 cc/24 hours/rat. Six rats were infused at a time so that the control and experimental animals were studied simultaneously. Temperature was constant at $26^\circ \pm 0.50^\circ\text{C}$.

At the end of the infusion period the blood was drained from the abdominal aorta by cannula. Heparin was used as an anticoagulant. The level of plasma amino acids was determined by the method of Hamilton and Van Slyke(2) and that of blood glucose by the method of Miller and Van Slyke(3).

Results. The conditions of each experiment and the results are shown in Table I. In agreement with earlier reports(1) insulin suppresses the rate of rise of plasma amino acids following evisceration in the rat. During a 3-hour period epinephrine failed to affect the level of plasma amino acids in either the presence or absence of insulin although in the presence of insulin and a glucose load of 70 mg/100 g of rat per hour the

TABLE I. Level of Plasma Amino Acids in Functionally Eviscerate Rats. Averages* and standard errors.

Exp.	Condition	Glucose load, mg/100 g rat/hr	Units insulin per 24 hr	Epinephrine, parts/million in 20 cc/24 hr	Hours	Concentration, mg %			
						Plasma amino acids		Blood glucose	
						Epinephrine	Control	Epinephrine	Control
1	Normal	0	0	0	0	10.08 ± .39	5.46 ± .91	109 ± 4.7	125 ± 4.6
2	Eviscerate	16	0	40	3	8.89 ± .24	9.48 ± .23	179 ± 11.2	179 ± 11.2
3	"	16	0	20	3	8.96 ± .37	9.12 ± .32	160 ± 8.55	160 ± 8.55
4	"	16	0	10	3	3.95 ± .30	9.44 ± .33	180 ± 9.89	167 ± 7.82
5	"	16	16	10	3	3.54 ± .16	3.98 ± .31	58 ± 3.48	48 ± 2.06
6	"	70	16	10	3	4.13 ± .12	3.77 ± .23	230 ± 13.33	79 ± 8.13
7	"	70	16	20	3	3.55 ± .09	4.11 ± .20	203 ± 16.7	86 ± 10.15
8	"	6	16	5	3	37.49 ± 1.34	3.87 ± .08	232 ± 8.79	73 ± 3.95
9	"	6	0	5	16	30.09 ± 2.38	35.16 ± 1.72	222 ± 27.93	193 ± 13.46
10	"	44	0	10	16	12.73 ± 1.05	30.3 ± 1.41	178 ± 17.09	222 ± 19.62
11	"	44	16	5	16	16.03 ± .69	11.67 ± .71	166 ± 24.74	145 ± 21.1
12	"	44	16	10	18	17.20 ± .73	12.88 ± 1.18	256 ± 35.3	137 ± 18.7
13	"	44	16	20	18		11.94 ± .51	389 ± 30.9	118 ± 14.35

* Each average represents 12-15 rats.

addition of epinephrine caused a striking rise in the level of blood glucose (experiments 6, 7, and 8). During a 16-hour period epinephrine failed to affect the level of plasma amino acids in eviscerated rats given glucose without insulin. When insulin was given with an increased glucose load the addition of epinephrine in doses of 10 and 20 parts per million caused a significant increase in the level of plasma amino acids (experiments 12 and 13) during a period of 18 hours.

Discussion. The effects of epinephrine and insulin upon the glucose tolerance of the eviscerate rat are in agreement with published data(4). Although large doses of epinephrine given with glucose and insulin for 18 hours have a positive effect upon the level of plasma amino acids in the eviscerate rat there is no clear relationship between the changes in the level of blood glucose and the changes in the level of plasma amino acids. The possible physiological significance of this extra-hepatic effect of large doses of epinephrine upon the level of plasma amino acids is not known. Adrenomedullated rats do not differ from normal rats with respect to the release of amino acids after evisceration(5).

Summary. Functionally eviscerated rats were given continuous intravenous injections of glucose with and without insulin, with and without epinephrine for periods of 3, 16, and 18 hours. Large doses of epinephrine given with glucose and insulin for 18 hours caused a significant rise in the level of plasma amino acids.

1. Russell, J. A., *Fed. Proc.*, 1955, v14, 696.
2. Hamilton, P. B., and Van Slyke, D. D., *J. Biol. Chem.*, 1943, v150, 231.
3. Miller, B. F., and Van Slyke, D. D., *ibid.*, 1936, v114, 583.
4. Ingle, D. J., and Nezamis, J. E., *Am. J. Physiol.*, 1949, v156, 361.
5. Frame, E. G., and Russell, J. A., *Endocrinol.*, 1946, v39, 420.

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Microbiological Studies on Growth Factor for *L. bifidus* var. *pennsylvanicus*.^{*} (21988)

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For *L. bifidus* var. *pennsylvanicus*(1) human milk furnishes a specific and essential growth factor (factors), named in short the "bifidus factor" (BF). In addition, this particular variant of *L. bifidus* is able to utilize only preformed pantethine but not pantothenic acid, even when the latter is offered in great excess. The growth of *L. bifidus* var. *pennsylvanicus* is further enhanced by a supplementary factor, present in human milk, cow's milk, insulin and pancreatic extract. For some mutants (or variants) this supplementary factor may act as an essential nutrient. The chemical characteristics and distribution of the supplementary factor appear to be closely related to those of streptogenin. The supplementary factor is different from the specific "bifidus factor"(2). Chemically the bifidus factor as it occurs in human milk belongs in the group of N-containing carbohydrates. In human milk the presence of a great variety of oligo- and polysaccharides has been demonstrated(3). Their total quantity in fresh human milk may be estimated to be around 0.4% which is by no means a negligible amount with respect to total solids. The bifidus factor in human milk is present in both low molecular dialyzable and high molecular, non-dialyzable form(4). The bifidus factor may be adsorbed from deproteinized human milk on charcoal and eluted by acetic acid(3). Such milk eluates contain also dialyzable and undialyzable fractions(4). The carbohydrates of human milk as obtained in the acetic acid eluates from charcoal adsorbates are composed of glucose, galactose, fucose and N-acetyl-D-glucosamine, in various combinations(3). Kuhn and his associates have found in human milk one non-N-containing triose (fucosido-lactose) and a group of N-containing oligosaccharides, consisting of a tetraose, 2 pentaoses and one hexaose. They have also determined the compo-

sition and exact structure of several of these oligosaccharides and of some of their breakdown products, such as of 2 bioses and 2 trioses obtained from the tetraose(5-10). In addition to the neutral oligo- and polysaccharides, human milk also contains acidic oligo- and polysaccharides, which occur again in dialyzable, low molecular and non-dialyzable, high molecular form. The acidic constituent of these saccharides has been isolated, chemically identified and tentatively named gynaminic acid(11). It is probably N-acetylneuraminic acid(11-13). Upon hydrolysis the oligo- and polysaccharides containing gynaminic acid yielded glucose, galactose, fucose and N-acetyl-D-glucosamine, in addition to gynaminic acid(11). The common link in all these N-containing oligo- and polysaccharides is the presence of N-acetyl-D-glucosamine. This is equally characteristic for all microbiologically active compounds obtained from other natural sources(14). As a matter of fact, N-acetyl-D-glucosamine, as well as N-acetyl-D-galactosamine and even inorganic ammonium salts have been found, in high concentration, active, probably as precursors of the bifidus factor(14). All chemically well defined oligosaccharides of human milk,[†] various disaccharides obtained from different sources and also through enzymatic or chemical synthesis, and finally a series of synthetic derivatives of N-acetyl-D-glucosamine have been tested microbiologically.

The present report summarizes the results of these studies.

Methods. The microbiological assays were carried out as previously described(1), with the semisynthetic basal medium(15,16) containing an enzymatic digest of casein ("NZ case peptone," Sheffield). The supplements were used in varying concentration and were

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[†]Most compounds from human milk, as well as from other sources, and a large number of synthetic compounds were kindly furnished by Professor Richard Kuhn, Heidelberg.

TABLE I. Saccharides Isolated from Human Milk.

No.	Rf (lactose)	Name	Composition	Microbiological activity, mg/unit		
				Ref.	Autoclaved	Sterile
V	1.0	Lactose			Inactive	Inactive
IV*	.73	N-free triose	Fucose Glucose > galactose	10		Inactive
IIIc*	.36	Lacto-N-tetraose	(Glucose > galactose > N-acetyl-D-glucosamine-galactose	3, 5, 6	2.4	1.7
	1.49	Lacto-N-biose I	N-acetyl-D-glucosamine-galactose (3-O- β -D- galactopyranosyl-N-acetyl-glucosamine)	6, 8	Inactive	1.5
IIc*	1.12	Lacto-N-biose II	Galactose > N-acetyl-D-glucosamine	6, 7	.6	.2
	.51	Lacto-N-triose I	Galactose > N-acetyl-D-glucosamine-galactose	6, 7	1.2	.75
	.70	Lacto-N-triose II	Glucose > galactose > N-acetyl-D-glucosamine	6, 7	.2	.2
IIId*	.27	Lacto-N-fuco-pentaose II	Fucose Glucose > galactose > N-acetyl-D-glucosamine > galactose	9	.75	.26
		Lacto-N-fuco-pentaose I	Glucose > galactose > N-acetyl-D-glucosamine > galactose	9	.3	.25
IIIe*	.11	Lacto-N-difuco-hexaose	Fucose Glucose > galactose > N-acetyl-D-glucosamine > galactose	9	1.1	.38
II	(gynaminic acid)	Rf				
		Acid saccharide II	Gynaminic acid; galactose; glucose; N-acetyl-D-glucosamine; fucose	11	.4	.4
		<i>Idem</i> I	Similar to II but higher in mol. wt	11	.15	.15

* Cf. Ref. 3.

TABLE II. Disaccharides of N-acetyl-D-glucosamine from Other Sources than Human Milk.

Name of compound	Origin	Ref.	Microbiological activity, mg/unit	
			Auto- claved	Sterile
4-0- β -D-galactopyranosyl-N-acetyl-D-glucosamine	From hog stomach mucin	17	.08-.1	.06
	Enzymatically synthesized	18, 19, 20	.08-.1	.06
	From meconium	21	.08-.1	.06
	Chemically synthesized	22	.08-.1	.06
3-0- β -D-galactopyranosyl-N-acetyl-D-glucosamine	Enzymatically synthesized	23	Inactive	1.5
6-0- β -D-galactopyranosyl-N-acetyl-D-glucosamine	Chemically synthesized	24	1.7	.95
	Enzymatically synthesized	18, 20	8	.90
6-0- β -D-(2'-deoxy-2'-acetamino)-glucopyranosyl-D-galactose	Chemically synthesized	25	.17	.17
6-0- β -D-(2'-deoxy-2'-acetamino)-glucopyranosyl-D-glucose	<i>Idem</i>	25	.10	.07
N-N'-diacetyl-chitobiose	From chitin	26	.8-1.8	1.2-2.2

either autoclaved with the medium or were added to it sterile (Seitz-filtered). The microbiological activity was expressed in mg per unit of the compound tested, related to the reference standard of skimmed human milk (1).

Results. Table I summarizes the results of the microbiological assays carried out with chemically well characterized compounds isolated from human milk. The Rf-values for the neutral oligosaccharides are based on that of lactose as unity (3), those for the acid saccharides are related to that of gynaminic acid (11). As expected, lactose and the N-free triose (fucosido-lactose) were microbiologically inactive. Great variation of activity was found for all N-containing oligosaccharides tested. Among the neutral oligosaccharides only lacto-N-triose II and lacto-N-fuco-pentaose I have shown high activity, regardless whether they were autoclaved with the medium or added to it sterile. High activity was also found for the acid saccharides, both in autoclaved and in sterile form. A few other compounds were highly active when used Seitz-filtered, but showed greatly reduced activity after autoclaving with the medium. It deserves special emphasis that 3-0- β -D-galactopyranosyl-N-acetyl-D-glucosamine, which is widely distributed in the N-containing oligosaccharides of human milk (6,8) exhibited no activity when autoclaved with the medium, and only very slight activity as sterile supplement. In contrast (Table II),

4-0- β -D-galactopyranosyl-N-acetyl-D-glucosamine showed the highest microbiological activity of all the naturally occurring derivatives of N-acetyl-D-glucosamine. This disaccharide, originally isolated from hog mucin (17), is present in human milk in only low concentration, especially when compared with 3-0- β -D-galactopyranosyl-N-acetyl-D-glucosamine. It has been obtained also from meconium (21), and through enzymatic (18,19) and chemical (22) synthesis, always with identical microbiological activity. In Table II several other disaccharides of N-acetyl-D-glucosamine are listed, some of them showing good microbiological potency.

In addition to the N-containing oligosaccharides, several synthetic derivatives of glucosamine were tested for microbiological activity (Table III). The α -anomers of the alkyl glycosides were found to be inactive. Unexpectedly very high activity was encountered with the β -alkyl compounds, in particular with the ethyl- and n-propyl-homologues. Even the benzyl and phenyl derivatives were strongly active. The activity of methyl-N-acetyl- β -D-glucosaminide was greatly enhanced (28) by the addition of the α -anomer and brought to the level of the pure ethyl- and n-propyl-compounds. On the basis of molecular weight the activity of these alkyl-derivatives of N-acetyl-D-glucosamine was comparable with that of 4-0- β -D-galactopyranosyl-N-acetyl-D-glucosamine. In this connection it may be added that in the course of

TABLE III. Synthetic Derivatives of N-Acetyl-D-Glucosamine.

Name of compound	Ref.	Microbiological activity, mg/unit	
		Auto-claved	Sterile
Methyl-N-acetyl- α -D-g*	27,28,29	Inact.	Inact.
Ethyl-N-acetyl- α -D-g	29	"	"
n-Propyl-N-acetyl- α -D-g	29	"	"
Benzyl-N-acetyl- α -D-g	†	1.5	—
Phenyl-N-acetyl- α -D-g	†	Inact.	—
Methyl-N-acetyl- β -D-g	28,29,30,33	.16-.2	—
Ethyl-N-acetyl- β -D-g	29,30	.04-.06	.05
n-Propyl-N-acetyl- β -D-g	29,30	.04-.06	.06
Isopropyl-N-acetyl- β -D-g	†	.100	.09
n-Butyl-N-acetyl- β -D-g	30†	.065	—
Benzyl-N-acetyl- β -D-g	30†	.095	—
Phenyl-N-acetyl- β -D-g	†	.05-.07	.05
N-formyl-D-g	†	2.5	—
Methyl-N-formyl- β -D-g	33†	2.5	—
Aniline-N-acetyl-D-g	†	Inact.	—
p-Anisidine-N-acetyl-D-g	†	"	—
p-Aminobenzoic acid ethyl ester N-acetyl-D-g	†	.7	—

* g = glucosamine.

† Kindly furnished by Dr. R. Kuhn, Heidelberg.
‡ " " " " Dr. S. Roseman, Ann Arbor.

the last 2 years the sensitivity of the strain of *L. bifidus* var. *pennsylvanicus* to pure N-acetyl-D-glucosamine autoclaved with the medium has shown a distinct, gradual deterioration from 2.3 mg per unit to 4.5-7.0 mg. No such reduction in sensitivity was noticed for the bifidus factor, as it is present in human milk, the active disaccharides, and the alkyl-derivatives of N-acetyl-D-glucosamine.

Discussion. In reviewing the results of the microbiological tests reported it is apparent that the various oligo- and polysaccharides and other derivatives of glucosamine have shown considerable variation in their activity as bifidus factor. The most potent compound of natural origin was the disaccharide 4-O- β -D-galactopyranosyl-N-acetyl-D-glucosamine. However, considering the relatively low concentration of this compound in human milk (5,6), it is difficult to assign to it the role of the specific bifidus factor. Further, the specificity of this disaccharide as bifidus factor is at variance with the fact that alkyl-glycosides of N-acetyl-D-glucosamine, especially the ethyl- and n-propyl derivatives show approximately the same high activity. Chemically the alkyl-glycosides and the disaccharide are not strictly comparable.

It is of additional interest that in previous

studies crude eluates from charcoal adsorbates of skimmed human milk or fractions obtained by alcohol precipitation of deproteinized milk(3,31) have often been found to have very high microbiological activity (about 250 γ per unit), comparable to the activity of above named simple derivatives of N-acetyl-D-glucosamine. Purified blood group substances with an activity of ca. 150 γ per unit were obtained from ovarian cyst fluid (32).

In the light of all these microbiological findings it is not possible yet to identify the bifidus factor with one specific compound.

Summary. 1. The results of microbiological assays on a large number of oligosaccharides obtained from human milk and on simple derivatives (disaccharides or simple synthetic compounds) of N-acetyl-D-glucosamine are reported. The high microbiological activity of 4-O- β -D-galactopyranosyl-N-acetyl-D-glucosamine and of ethyl- and n-propyl-N-acetyl- β -D-glucosamine has not been exceeded by any other compound tested. 2. The "true" specific bifidus factor has either still to be found or several related compounds may act as precursors or in synergistic combination.

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1. György, P., Norris, R. F., and Rose, C. S., *Arch. Biochem. and Biophys.*, 1954, v48, 193.
2. György, P., and Rose, C. S., *J. Bact.*, 1955, v69, 483.
3. Gauhe, A., György, P., Hoover, J. R. E., Kuhn, R., Rose, C. S., Ruelius, H. W., and Zilliken, F., *Arch. Biochem. and Biophys.*, 1954, v48, 214.
4. György, P., Hoover, J. R. E., Kuhn, R., and Rose, C. S., *ibid.*, 1954, v48, 209.
5. Kuhn, R., Gauhe, A., and Baer, H. H., *Berichte*, 1953, v86, 827.
6. ———, *ibid.*, 1954, v87, 289.
7. ———, *ibid.*, 1954, v87, 1187.
8. Kuhn, R., Baer, H. H., and Gauhe, A., *ibid.*, 1954, v87, 1553.
9. Kuhn, R., *Angew. Chemie.*, 1955, v67, 184.
10. Kuhn, R., Baer, H. H., Gauhe, A., *Ber.*, 1955, v88, 1135.
11. Zilliken, F., Braun, G. A., and György, P., *Arch. Biochem. and Biophys.*, 1955, v54, 564.
12. Hoover, J. R. E., Braun, G. A., and György, P., *ibid.*, 1953, v47, 216.
13. Klenk, E., and Faillard, H., *Z. physiol. Chem.*,

1954, v298, 230.

14. György, P., Kuhn, R., Rose, C. S., and Zilliken, F., *Arch. Biochem. and Biophys.*, 1954, v48, 202.

15. Norris, R. F., Flanders, T., Tomarelli, R. M., and György, P., *J. Bacteriol.*, 1950, v60, 681.

16. Hassinen, J. B., Durbin, G. T., Tomarelli, R. M., and Bernhart, F. W., *ibid.*, 1951, v62, 771.

17. Tomarelli, R. M., Hassinen, J. B., Eckhardt, E. R., Clark, R. H., and Bernhart, F. W., *Arch. Biochem. and Biophys.*, 1954, v48, 225.

18. Zilliken, F., Smith, P. N., Rose, C. S., and György, P., *J. Biol. Chem.*, 1954, v208, 299.

19. Zilliken, F., Smith, P. N., Tomarelli, R. M., and György, P., *Arch. Biochem. and Biophys.*, 1955, v54, 398.

20. Zilliken, F., Smith, P. N., Rose, C. S., and György, P., *J. Biol. Chem.*, in press.

21. Kuhn, R., and Kirschenbohr, W., *Ber.*, 1954, v87, 560.

22. ———, *ibid.*, 1954, v87, 1547.

23. Alessandrini, A., Schmidt, E., Zilliken, F., and

György, P., unpublished data.

24. Kuhn, R., and Baer, H. H., unpublished data.

25. Kuhn, R., and Kirschenbohr, W., *Ber.*, 1954, v87, 384.

26. Zilliken, F., Braun, G. A., and György, P., *J. Am. Chem. Soc.*, 1955, v77, 1296.

27. Kuhn, R., Zilliken, F., and Gauhe, A., *Ber.*, 1953, v86, 466.

28. Rose, C. S., Kuhn, R., Zilliken, F., and György, P., *Arch. Biochem. and Biophys.*, 1954, v49, 123.

29. Zilliken, F., Rose, C. S., Braun, G. A., and György, P., *ibid.*, 1955, v54, 392.

30. Kuhn, R., and Kirschenbohr, W., *Ber.*, 1953, v86, 1331.

31. Brown, G. A., Zilliken, F., and György, P., unpublished data.

32. Springer, G. F., Rose, C. S., and György, P., *J. Lab. and Clin. Med.*, 1954, v43, 532.

33. Kuhn, R., and Baer, H. H., *Ber.*, 1953, v86, 724.

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Effects of β -Sitosterol and Ferric Chloride On Accumulation of Cholesterol in Mouse Liver.* (21989)

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Since repeated experimental evidence has associated high cholesterol levels with atherosclerosis, various attempts have been made to lower blood and organ cholesterol content. The effects of several substances on cholesterol absorption have been studied using various experimental animals. Soybean sterols, consisting mainly of β -sitosterol, have been shown to decrease the accumulation of dietary cholesterol(1-5). Studies on dihydrocholesterol treated animals have shown that this substance is also effective in reducing cholesterol absorption(6-8). Moreover, it has been found that, in the cockerel, precipitation of bile acids by ferric chloride results in a reduction of blood cholesterol(9,10). On the other hand, in the case of the mouse and rat, evidence has been produced that soybean sterols fail to

lower organ and blood cholesterol(11,12).

The object of this experiment is to study: first, the effectiveness of β -sitosterol in prevention of liver cholesterol accumulation; and second, the effectiveness of ferric chloride in a mammalian species.

Methods. Exp. I. Dietary β -sitosterol. Webster strain female albino mice weighing 15 to 20 g were used. Throughout the experiment all mice were fed *ad lib.* a basal fat-free, cholesterol-free diet, described in a previous paper(7), supplemented by adequate amounts of vit. A and D. After a 2-week preliminary period on this basal diet, the mice were divided into 7 groups. The controls, Group I, were continued on the basal diet. For the other 6 groups the basal diet was supplemented with 1% cholesterol. Cholic acid was also added to the various diets as follows: Groups II and V received 0.25%; Groups III and VI, 0.5%; and Groups IV and VII, 1%. In addition, diets for Groups V, VI, and VII contained

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TABLE I. Effect of β -Sitosterol and Cholic Acid on Total Mouse Liver Cholesterol.

Group and diet	No. of mice	Additions to basal fat-free diet (%)			Total cholesterol in liver (mg)	
		Cholesterol	Cholic acid	β -sitosterol	Per organ†	Per g body wt‡
I	12	—	—	—	10.6 \pm 2.1*	.53 \pm .11*
II	10	1	.25	—	36.1 \pm 6.3	1.88 \pm .28
III	9	1	.5	—	47.1 \pm 9.1	2.40 \pm .52
IV	10	1	1.0	—	48.0 \pm 13.3	2.20 \pm .60
V	10	1	.25	2.5	30.0 \pm 6.1	1.37 \pm .23
VI	11	1	.5	"	24.9 \pm 6.4	1.27 \pm .33
VII	9	1	1.0	"	34.2 \pm 6.8	2.04 \pm .41

* Stand. dev.

† Significance of the difference in results of the following groups: (a) II & V, $P \cong 0.04$;(b) III & VI, $P < 0.01$; (c) IV & VII, $P < 0.01$.‡ Significance of the difference in results of the following groups: (a) II & V, $P < 0.01$;(b) III & VI, $P < 0.01$; (c) IV & VII, $P \cong 0.5$.

2.5% β -sitosterol. All diets were fed *ad lib.* for a 3-week experimental period, during which food consumption for the 7 groups varied from 2.9 g to 3.4 g per mouse per day. After the experimental feeding, the mice were sacrificed, and the livers removed, weighed, and quick frozen. Total liver cholesterol was assayed by a procedure similar to the one used in our previous study(7).

Exp. II. Dietary ferric chloride. National Institute of Health strain female albino mice weighing 15 to 20 g were used. As in Exp. I, all animals were fed *ad lib.* a basal fat-free, cholesterol-free diet for an initial 2-week period. The mice were then divided into 5 groups. For all groups, the basal diet was supplemented with 1% cholesterol and 0.5% cholic acid. In addition iron, as ferric chloride, was added to the diets as follows: Group I received no iron; Group II received 0.05%; Group III, 0.5%; Group IV, 1%; and Group V, 2%. After being fed *ad lib.* on these experimental diets for 3 weeks, the mice were sacrificed, and the livers quickly removed, weighed and frozen. Total cholesterol determinations were made as in Exp. I.

Results. Table I presents the data on the effect of β -sitosterol and cholic acid on the accumulation of mouse liver cholesterol. As can be seen, increasing the dietary cholic acid increased the concentration of liver cholesterol up to a maximum value attained in Group III. Comparison of Groups II and V, III and VI, and IV and VII shows the effectiveness of β -sitosterol in preventing liver cholesterol buildup. It is to be noted that β -sitosterol is most

effective in the presence of 0.5% cholic acid. However, in none of these groups did total cholesterol concentration approach the control values.

Results of simultaneous feeding of cholesterol and ferric chloride are shown in Table II. The data indicate that ferric chloride failed to decrease cholesterol accumulation. In fact, increasing the concentration of ferric chloride seemed to increase the cholesterol level. Even though comparison of Groups I and II may suggest a slight decrease, the significance of this difference is doubtful in view of the large standard deviation.

Discussion. From the data in Table I, it can be seen that β -sitosterol is effective in decreasing liver cholesterol accumulation under certain conditions. However, comparison of the present results with those obtained in an identical experiment(7) with dihydrocholesterol, shows that, in the presence of equal amounts of cholic acid, dihydrocholesterol is more effective than β -sitosterol.

Data on the ferric chloride experiment indicate that this compound does not reduce dietary cholesterol accumulation in liver. This finding does not coincide with that of Siperstein *et al.*(9), who postulated that ferric chloride binds the bile acids in the intestinal tract, thereby suppressing cholesterol absorption. However, it must be noted that two major differences exist between their experiment and the present one: First, Siperstein *et al.* experimented on cockerels, while in our experiment, mice were used; and second, they studied blood cholesterol levels, while our data

TABLE II. Effect of Ferric Chloride on Total Mouse Liver Cholesterol.

Group and diet	No. of mice	Additions to basal fat-free diet (%)			Total cholesterol in liver (mg)	
		Cholesterol	Cholic acid	Iron as FeCl ₃	Per organ	Per g body wt
I	7	1	.5	—	56.3 \pm 11.7*	2.81 \pm .65
II	8	1	.5	.05	43.9 \pm 10.4	2.22 \pm .38
III	7	1	.5	.5	53.5 \pm 11.5	2.54 \pm .46
IV	7	1	.5	1.0	68.6 \pm 17.4	3.36 \pm .65
V	7	1	.5	2.0	67.6 \pm 7.6	3.76 \pm .50

* Stand. dev.

are measurements of liver cholesterol concentrations. The differences found in these experiments may be explained by the hypothesis that ferric ions have a toxic action on liver cells, which prevents the normal distribution of cholesterol between the blood stream and the liver. In agreement with Siperstein *et al.*, we found that ferric chloride, fed in concentrations of 2% or more, proved to be highly toxic and, in some cases, fatal after a few days. The possible physiological after-effects of even small amounts of this compound cannot be overlooked.

Summary. 1. Increasing the cholic acid content of a diet rich in cholesterol, significantly increased total liver cholesterol. 2. The addition of β -sitosterol to diets containing cholic acid and cholesterol, decreased total liver cholesterol. 3. Addition of ferric chloride, in varying amounts, to diets rich in cholesterol and cholic acid, resulted in practically no change in total cholesterol content of mouse liver.

- Peterson, D. W., *Proc. Soc. Exp. Biol. and Med.*, 1951, v78, 143.
- Peterson, D. W., Shneour, E., Peek, N., and Gaffey, H., *J. Nutrition*, 1953, v50, 191.
- Pollak, O., *Circulation*, 1953, v7, 696.
- Hernandez, H., Peterson, D. W., Chaikoff, I. L., and Dauben, W., *Proc. Soc. Exp. Biol. and Med.*, 1953, v83, 498.
- Alfin-Slater, R. B., Wells, A. F., Aftergood, L., Melnick, D., and Deuel, H. J., *Circulation Research*, 1954, v2, 471.
- Rosenman, R. H., Byers, S. O., and Friedman, M., *ibid.*, 1954, v2, 45.
- Beher, W. T., and Anthony, W. L., *J. Nutrition*, 1954, v52, 519.
- Siperstein, M. D., Nichols, C. W., and Chaikoff, I. L., *Circulation*, 1953, v7, 37.
- , *Science*, 1953, v117, 386.
- Siperstein, M. D., Chaikoff, I. L., and Reinhardt, W., *J. Biol. Chem.*, 1952, v198, 111.
- Schettler, G., *Klin. Wschr.*, 1948, v26, 566.
- Rosenman, R. H., Byers, S. O., and Friedman, M., *Circulation Research*, 1954, v2, 160.

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Identification by Fluorescent Antibody of Developmental Forms of Psittacosis Virus in Tissue Culture.* (21990)

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Bedson(1) has described a developmental cycle for psittacosis elementary bodies in the cytoplasm of host cells. The cytologic studies (1-6) proved that the intermediate developmental forms such as initial bodies and plaques are regularly associated with the propagation of virus and are structural evidence of cellular infection. Morphologic observations have failed to establish whether the large structures are secondary reaction products or multiplying virus. Electron micrography has so far produced only equivocal evidence(7-9). The present study was undertaken with the hope that the immunocytochemical method for intracellular identification of antigen described by Coons and his co-workers(10) might determine the nature of the inclusion bodies.

Materials and methods. *Virus:* The psittacosis 6 BC strain was generously provided by Dr. Herbert Morgan, University of Rochester School of Medicine, and was maintained by yolk sac passage in embryonated eggs. A 10% yolk sac suspension with an LD₅₀ titer of 10⁻⁸ was used. Albany strain mice were employed. Infectivity tests were performed in young adults (8-12 g). Pregnant mice 60-80 days old were the source of mouse embryos. Groups of 5 mice were inoculated intracerebrally with 0.03 ml of undiluted supernates of lightly centrifuged ground tissue suspension, then observed for 21 days. *Tissue culture:* Pregnant mice, approximately at the end of the period of gestation, were sacrificed by ether anesthesia. Embryonic livers were removed aseptically and minced with

detachable knife blades into 1- x 2-mm pieces. Five pieces were planted on a 10- x 20-mm cover glass and 5 were planted on a 10- x 10-mm glass fiber mesh(11) placed in the flat bottom of a Leighton tube (Microbiological Associates, Bethesda, Md.). Approximately 0.1 ml of horse serum was added to the explants. Preparations were incubated at 40°C for 20 minutes, after which nutrient fluid(12) (beef amniotic fluid 85%, beef embryo extract 5%, horse serum 10%, 50 µg/ml of crystalline dihydrostreptomycin sulfate (Merck)) was added, 0.5 ml per tube. Cultures were incubated at 35°C for 5 days prior to use. *Fluorescent-antibody technic:* The indirect method of Weller and Coons(13), and Coons, Leduc, and Connolly(14) was applied. Fluorescein isocyanate was synthesized and conjugated to the pseudoglobulin fraction of antihuman gamma globulin horse serum‡ according to methods described by Coons and Kaplan(10) with minor alterations. For specific combination with intracellular antigen, human antipsittacosis serum (No. V54-2029) was used. The 50% unit titer was 1285 in a complement-fixation test against a phenolized 10% yolk sac antigen. A human serum (No. V54-1766) was the control. Nonspecific staining was eliminated by absorption of the conjugate with whole mouse embryo powder and subsequent dilution with buffered saline (sodium chloride solution, phosphate buffered to pH 7.0). Cover-glass preparations from inoculated and uninoculated groups were removed and treated as described by Weller and Coons(13). Stained cultures were examined for the presence of intracellular antigen under the fluorescence microscope. Its light source was a 1000-watt General Electric water-cooled mercury vapor lamp (type A-H6). The beam was condensed

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‡ Generously supplied by Dr. David Gitlin, Children's Hospital, Boston.

by a quartz lens (61.5 mm diameter, 59.5 mm focal length). Infrared and visible light was filtered by passing the beam through 25% CuSO₄ in a water-cooled cell in which a Corning glass filter (#5840, 1/2 standard thickness) was immersed. Slides were viewed through a dark-field microscope with a Wratten 2 B gelatin filter in the ocular. Photomicrographs were taken with a Zeiss Miflex camera and super ortho press film. Exposure for 400X magnification was 3 minutes. Films were tray-developed in DK-50 for 5 minutes at 20°C.

Results. Specificity of fluorescent antibody technic: In preliminary experiments, infected and uninfected explants were exposed for 20 minutes to 1:10 dilutions, respectively, of human antipsittacosis serum and of human serum which did not react in the complement-fixation test with psittacosis antigen. Cultures on cover glasses were washed for 10 minutes with buffered saline, then overlaid for 20 minutes with a 1:10 dilution of fluorescein conjugate. Cultures were again washed for 10 minutes and mounted as described by Weller and Coons(13). Brilliant yellow-green fluorescence was observed in the cytoplasm of infected cells treated with human antipsittacosis serum. Uninfected explants exposed to human antipsittacosis serum and infected explants treated with nonreacting human serum showed blue-gray autofluorescence.

Relation of inoculation dose to specific fluorescence and infective virus: Cultures with good outgrowth were divided into 3 groups inoculated respectively with 0.05 ml of 10⁻¹, 10⁻³, and 10⁻⁵ dilutions in nutrient fluid of yolk sac suspension. At varying times (Table I) preparations of each group were treated in the following manner: 1. Cover-glass preparations were washed free of extracellular antigen in 3 changes of buffered saline. Exposure of infected cultures to human antipsittacosis serum was followed by staining with fluorescent antibody. Time factors for the staining procedure were substantially the same for all preparations. 2. Glass fiber patches carrying infected explants were removed from Leighton tubes and washed in 6 changes of buffered saline to minimize contamination by residual antigen, then ground

TABLE I. Comparison of Appearance of Specific Fluorescence and Presence of Infective Virus at Various Time Intervals following Inoculation of Different Doses of Virus.

Dil. of virus inoc.	Neg. * log of LD ₅₀ titer	Time after inoculation—specific fluorescence†—infective virus†											Avg time until death (mice), days	
		0 min.	10 min.	1 hr	3 hr	5 hr	8 hr	12 hr	16 hr	20 hr	24 hr	48 hr		72 hr
10 ⁻¹	5.3	—	±	+	+	+	+	+	+	+	+	+	+	3-6
		4/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5	
10 ⁻³	2.5	—	—	—	—	—	±	+	+	+	+	+	+	5-14
		NT	NT	0/5.	0/5	2/5	3/5	5/5	2/5	4/5	3/5	5/5	5/5	
10 ⁻⁵	<1.0	—	—	—	—	—	—	—	—	—	—	±	+	20
		NT	NT	NT	NT	NT	1/5	0/5	0/5	0/5	0/5	0/5	0/5	

* LD₅₀ titer of ground tissue and nutrient fluid at 0 time.

† Grading of results explained in text.

‡ Presence of infective virus in washed tissue explants; numerator = No. of dead mice; denominator = No. of mice inoculated. NT = Not tested.

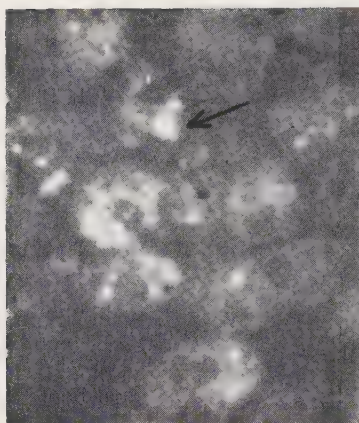


Fig. 1

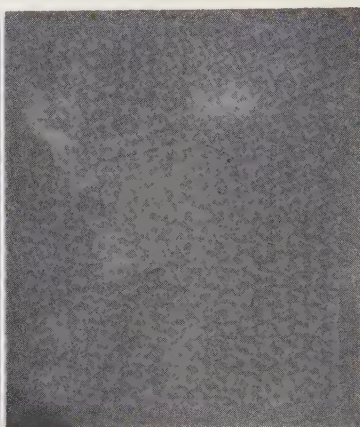


Fig. 2



Fig. 3

FIG. 1. Mouse embryo liver cells infected with 10^{-1} dose of virus; stained by fluorescent technic 72 hr after inoculation. Arrow points to plaque. $400\times$.

FIG. 2. Mouse embryo liver cells infected with 10^{-5} dose of virus; stained by fluorescent technic 72 hr after inoculation. $400\times$.

FIG. 3. Mouse embryo liver cells infected with 10^{-1} dose of virus; stained by Giemsa's method 72 hr after inoculation. Arrow points to plaque. $400\times$.

in mortars with alundum and 0.5 ml of nutrient fluid per 5 explants. After light angle centrifugation supernates were tested for infectivity in mice. In 10 different experiments, approximately 260 cover-glass preparations, carrying 5 explants each, were examined for presence of specific fluorescence at different intervals postinoculation. Results were graded from — to +++++, — indicating absence of specific fluorescence; \pm a trace; + involvement of 5%; ++ 6-25%; +++ 26-75%; and ++++ the presence of specific fluorescence in more than 75% of the outgrowing cells. With the heaviest virus inoculum (10^{-1}), intensity of specific cytoplasmic fluorescence was moderate early and became marked 16 hours following inoculation and remained intense throughout the experiment (Fig. 1). With a medium (10^{-3}) dose of virus, two distinct features were noted: The appearance of specific fluorescence was delayed and its distribution was focal, involving 5-25% of outgrowing cells. The intensity was weak for the first 16 hours, and became moderate for the rest of the experiment. Negative results were obtained when the 10^{-5} dilution was used (Fig. 2).

Tests for the presence of virus in washed explants indicated that it was present in all groups inoculated with the 10^{-1} dilution. The

average time to death decreased in the 10^{-1} and 10^{-3} groups as the experiment progressed, evidence of the multiplication of the virus.

Identification of antigen in developmental forms. In this series of experiments, the morphology of cultures stained with fluorescent antibody was compared with Giemsa[§]-stained cover-glass preparations. Diffuse fluorescence was observed in the early stages, 1, 3, and 5 hours postinoculation. Stippling, i.e., the appearance of tiny fluorescent granules, distributed evenly throughout the cytoplasm, occurred as early as 8 hours following inoculation. It compared well with purple red staining elementary bodies and blue staining larger initial bodies in the cytoplasm of the cells stained by Giemsa's method. Concurrent with the blue staining vesicles and plaques at 12- and 16-hour intervals, larger fluorescing bodies were observed, sometimes eccentrically located in the cytoplasm. The size of the inclusion bodies in the Giemsa preparations continued to be much the same as in the fluorescent-antibody preparations throughout the growth cycle of the virus in cultures inoculated with the moderate (10^{-3}) and heavy (10^{-1}) doses (Fig. 3). Cultures in-

[§] Giemsa stock solution, Merck and Company, Darmstadt, Germany.

oculated with the low (10^{-5}) dose, which showed no specific fluorescence, likewise developed no inclusion bodies detectable by Giemsa stain.

Discussion. By the immunocytochemical criterion described it was shown that antigen was regularly present within the developmental forms of psittacosis virus and that the inclusion bodies can be considered to be virus colonies rather than reaction products of infected cells. Bedson and Gostling(15) have suggested that multiplication of psittacosis virus is initiated as soon as elementary bodies are introduced into a susceptible tissue. This view is substantially supported by our *in vitro* observation that virus can be located within the cytoplasm of a few cells as early as one hour after inoculation. Whether the early intracellular appearance of antigen signifies the presence of infective virus has not been settled, since repeated washing of explants failed to remove traces of residual virus. In groups inoculated with a moderate dose, however, the early stages were characterized by lack of specific fluorescence and negative infectivity tests. With the appearance of intracellular fluorescence, infective virus was also demonstrated.

A large dose of virus infected 75% or more of outgrowing cells within the first 16 hours, whereas a smaller dose produced only a focal distribution of specific fluorescence, leaving 75% or more of outgrowing cells uninfected.

Weiss(5) has described intranuclear granules resembling elementary bodies 7 to 10 days after infection of experimental animals, and considered the possibility that the virus had invaded the nuclei. In our experiments specific fluorescence appeared within the cytoplasm alone during the 72-hour observation period. The various stages of cellular division were not correlated with the detection of specific fluorescence. No attempt was made to examine for extracellular forms of virus(5), since the search for intracellular antigen necessitated frequent washing of cultures to minimize residual virus. The fluorescent-antibody technic proved to be more sensitive than Giemsa's differential stain for detection of virus provided the inoculation dose was large. Giemsa staining failed to disclose with cer-

tainty intracellular inclusions at 1- and 3-hour intervals following inoculation, whereas the immunocytochemical stain detected intracellular antigen earlier and in greater quantity throughout the experiment. The difference may have been affected by the difference in method of fixation, formalin(16) being used for the Giemsa preparations, while air drying (1 hour at 37°C) followed by acetone was employed for the fluorescent-antibody studies. Antigen might be lost during the former treatment. Numerous observations of empty vesicles within the cytoplasm of Giemsa-stained cells suggested this might have been the case. A similar loss of antigen may have occurred during the studies of Kurotchkin *et al.*(7) who reported disintegration of large structures following treatment with ether for 5 minutes and reasoned that inclusion bodies represent secondary reaction products, perhaps of lipid nature, rather than virus colonies.

Summary. 1. Developmental forms of psittacosis virus have been studied in mouse embryo liver tissue cultures *in vitro* by the fluorescent-antibody technic. 2. Inoculation of a large dose of virus resulted in the intracellular appearance of antigen in most of the outgrowing cells within 16 hours. A moderate dose of virus resulted in the focal distribution of specific fluorescence. When a small dose of virus was inoculated, no specific fluorescence was detected. 3. The evidence indicates that virus antigen is present in the developmental forms within the cytoplasm of infected cells throughout the various stages that make up the growth cycle of psittacosis virus.

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1. Bedson, S. P., *Brit. J. Exp. Path.*, 1932, v13, 65.
2. Bedson, S. P., and Bland, J. O. W., *ibid.*, 1932, v13, 461.
3. Bedson, S. P., *ibid.*, 1933, v14, 267.
4. Bland, J. O. W., and Canti, R. G., *J. Path. and Bact.*, 1935, v40, 231.
5. Weiss, E., *J. Inf. Dis.*, 1949, v84, 125.
6. Rake, G., and Jones, H. P., *J. Exp. Med.*, 1942, v75, 323.
7. Kurotchkin, T. J., Libby, R. L., Gagnon, E.,

and Cox, H. R., *J. Immunol.*, 1947, v55, 283.

8. Rake, G., Rake, H., Hamre, D., and Groupé, V., *PROC. SOC. EXP. BIOL. AND MED.*, 1946, v63, 489.

9. Gaylord, W. H., Jr., *J. Exp. Med.*, 1954, v100, 575.

10. Coons, A. H., and Kaplan, M. H., *ibid.*, 1950, v91, 1.

11. Warner, D., Hanawalt, C., and Bischoff, F., *J. Nat. Cancer Inst.*, 1949, v10, 67.

12. Enders, J. F., *PROC. SOC. EXP. BIOL. AND MED.*,

1953, v82, 100.

13. Weller, T. H., and Coons, A. H., *ibid.*, 1954, v86, 789.

14. Coons, A. H., Leduc, E. H., and Connolly, J. M., *J. Exp. Med.*, 1955, v102, 49.

15. Bedson, S. P., and Gostling, J. V. T., *Brit. J. Exp. Path.*, 1954, v35, 299.

16. Herzberg, K., *Zentralbl. f. Bakt.*, 1954, v160, 481.

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Protection Against Tourniquet Shock Afforded by Parabiosis.* (21991)

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When rats are placed in parabiosis one of them may thereby be protected against otherwise fatal consequences. It has been shown for example, that survival of one is prolonged after total nephrectomy(1), a lethal dosage of x-rays(2,3) or bilateral adrenalectomy(4), when the co-twin is normal. Similarly the usually severe diabetes following either pancreatectomy(5) or toxic doses of alloxan(6) is either minimized or abolished by the presence of a normal partner. Recently we have reported that when one of a normal pair of parabiotic rats is subjected to transection of the spinal cord just caudad to C7, the operated rat develops erythremia, with increased hemoglobin, erythrocyte count and hematocrit, whereas the intact co-twin concurrently develops a rapidly fatal anemia(7). This study was interpreted to indicate that the capillary hypotension induced on one side of the capillary anastomosis by spinal transection led to a decrease in the amount of blood exchanged from operated to intact, while the amount traversing the vascular bed from intact to operated remained normal. These changes, typical of spontaneous parabiosis intoxication (8), suggest the latter to be due to a shock-like state in one of the partners.

The present study was undertaken to determine whether the disparity in blood transfer

could also be induced by subjecting one of a pair to tourniquet shock, or whether the usually lethal effects of this intervention would be modified by the presence of a normal twin.

Materials and methods. The animals used in these studies were females of the Holtzman strain. Thirteen single animals weighing 100-139 g served as controls, and 10 pairs of parabionts, in union for 2 weeks and together weighing 200-280 g, constituted the parabiotic group. Parabiosis was performed essentially by the method of Bunster and Meyer(9). Tourniquets were applied to both hind limbs of the singles and to both hind limbs of one of the partners of each pair. The method was similar to that described by Rosenthal(10) and Stopack(11). A #8 elastic band was looped 5 times around a hollow tube of 16 mm diameter, each hind limb was then drawn through the tube and the elastic ligature slipped off so as to constrict the limb as close to the body as possible. The ligatures were removed 5 hours later. Rectal temperature, erythrocyte count, hematocrit and hemoglobin determinations were taken on all animals prior to ligation, and at 12, 36, and 48 hours after its removal, where survival permitted. Purina Laboratory Chow and tap water were available to the rats at all times.

Results. Of the 13 single rats to which the tourniquets were applied, 5 died within 12 hours of removal of the ligatures and all but one had succumbed by 48 hours thereafter.

* Supported by a grant-in-aid from the American Cancer Society upon recommendation from the Committee on Growth of the National Research Council.

TABLE I. Effect of Tourniquet Shock on Single and Parabiotic Rats.

A—Single rats									
Time	No. rats	Rectal temp., °C		Erythrocytes, millions cu/mm		Hemoglobin, g/100 cc		Hematocrit	
Before tourniquet	13	38.0 ± 1.4*		7.50 ± .30		14.9 ± .3		42 ± 1.2	
12 hr after removal	8	29.4 ± 1.2		10.43 ± .36		20.1 ± .5		62 ± 1.0	
36 " " "	2	28.8 ± .8		11.15 ± .83		21.5 ± .9		65 ± 3.1	
48 " " "	1	38.4		9.80		15.3		51	
B—Parabiotic pairs									
Time	No. pairs	Rectal temp., °C		Erythrocytes, million cu/mm		Hemoglobin, g/100 cc		Hematocrit	
		Left†	Right	Left	Right	Left	Right	Left	Right
Before tourniquet	10	38.2 ± .3	38.2 ± .3	7.57 ± .26	8.15 ± .39	14.6 ± .3	15.0 ± .6	42 ± 1.2	44 ± 1.7
12 hr after†	10	37.5 ± .2	37.7 ± .2	9.12 ± .39	8.05 ± .50	16.4 ± .2	14.6 ± .2	50 ± 1.1	43 ± 1.6
36 " "	10	38.0 ± .2	37.9 ± .2	7.09 ± .32	6.69 ± .59	13.0 ± .3	12.3 ± .9	37 ± 1.4	36 ± 2.3
48 " "	10	38.2 ± .1	38.2 ± .2	7.02 ± .42	6.66 ± .46	12.8 ± .4	12.6 ± .8	37 ± 1.6	38 ± 2.4
± S.E. of the mean.		† Animal with tourniquet applied.				‡ After removal.			

Rectal temperatures declined rapidly, and there occurred a marked hemoconcentration as evidenced by increase in the red-cell count, hemoglobin and hematocrit which was progressive until death. The results are given in Table IA.

The behavior of parabiotic rats was in marked contrast. Following removal of the tourniquets from the limbs of the one animal, there occurred a transient hemoconcentration at 12 hours, reflected in the hemogram, but no alteration in rectal temperature. However, at the 12-hour interval hemoconcentration was not nearly as marked in these as in single animals similarly treated, and whereas it was progressive in the latter it promptly reverted towards normal in the parabionts. Further, although the edema of the limbs was as marked in parabionts as in singles following ligature removal, and indeed in one the limbs amputated 4 days thereafter, none of them succumbed. The hemograms of the untreated partners indicated an anemic tendency. This was detectable at the 36-hour interval, was still present at 48 hours, and recovered slowly thereafter. Inasmuch as each of the pairs had tended to stabilize within 48 hours, no further blood determinations were made, although survival was followed for 2 weeks. The results are summarized in Table IB.

Discussion. Since the chief connection between the twins is vascular, and since the recovery from transient hemoconcentration in

the injured rat was accompanied by hemodilution in the untreated partner, it seems reasonable to attribute the protective effect of parabiosis to the availability of the normal partner's blood to the injured twin. The magnitude of this interchange was sufficient to induce changes in the hemogram of the untreated animal in most cases although not to the same degree as has been true of untreated rats, attached to twins subjected to spinal transection, which invariably succumb. It would seem likely that the erythrocyte and fluid reserves available to the untreated twin are often sufficient to enable it to maintain an almost normal hemogram in the face of demands by the damaged twin. Only when the reserves are strained, as when a normal animal is attached to a twin with a spinal transection(7), to a dying or dead partner (12), or perhaps when one is attached to the hyperemic member of a pair with spontaneous parabiosis intoxication(12), is the resulting demand upon the vascular fluids of the normal partner sufficiently severe as to induce a fatal anemia. This is believed to be due to whole blood transfusion of one rat by its partner, with the latter undergoing hemodilution in an effort to sustain the declining blood volume as discussed elsewhere(13).

Summary. 1. Elastic ligatures applied to both hind limbs of young female rats for 5 hours produced shock and death in 12 of 13 single rats; a marked and progressive decrease

in body temperature and an increase in hemoconcentration invariably occurred in those which succumbed. 2. Tourniquets applied similarly to the limbs of one partner failed to produce a single death in 10 pairs of parabionts. A transient hemoconcentration was observed in the injured partner at 12 hours although rectal temperatures remained stable. Anemia occurred in the untreated member of many of the pairs, and is believed to reflect transfusion of the shocked rats, with compensatory hemodilution in an effort to sustain the blood volume.

1. Grollman, A., and Rule, C., *Am. J. Physiol.*, 1943, v138, 587.
2. Brecher, G., and Cronkite, E. P., *PROC. SOC. EXP. BIOL. AND MED.*, 1951, v77, 292.
3. Finerty, J. C., Binhammer, R., and Schneider, M., *Texas Rep. Biol. and Med.*, 1952, v10, 496.

4. Mayeda, T., *Deutsche Z. f. Chir.*, 1921, v167, 295.
5. Fels, E., and Foglia, V. G., *Rev. Soc. argent. Biol.*, 1949, v25, 62.
6. Weitze, M., and Riggins, G. M., *Anat. Rec.*, 1954, v118, 857.
7. Hall, C. E., and Hall, O., *Fed. Proc.*, 1955, v14, 68.
8. Chute, R. N., and Sommers, S. C., *Blood*, 1952, v7, 1005.
9. Bunster, E., and Meyer, R. K., *Anat. Rec.*, 1933, v57, 339.
10. Rosenthal, S. M., *U. S. Pub. Health Rep.*, 1943, v58, 1429.
11. Stopack, J. H., *Am. J. Physiol.*, 1953, v175, 99.
12. Hall, C. E., and Hall, O., *Am. J. Physiol.*, (in press).
13. ———, *J. Exp. Med.* (in press).

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Inhibition of Lipid Synthesis by Alpha-Phenyl-N-Butyrate and Related Compounds. (21992)

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Cottet *et al.* have reported that feeding alpha-phenylbutyrate (phenylethylacetate) can lower serum cholesterol levels in the normal rat(1,2) and in patients with various forms of hypercholesterolemia(3). Since the drug did not prevent hypercholesterolemia in cholesterol-fed rats(1), it presumably does not act by inhibiting intestinal absorption; but the mechanism of action remains to be established. The present studies show that *in vitro* the drug strikingly inhibits synthesis of both cholesterol and fatty acids from sodium acetate-1-C¹⁴.

Methods. Male Sprague-Dawley rats maintained on Purina rat chow to the time of sacrifice were decapitated and livers rapidly removed and chilled. 350-450 mg liver slices made with the Stadie-Riggs microtome were incubated with sodium acetate-1-C¹⁴ at 37°C in Krebs-Ringer phosphate buffer, pH 7.4, from which Ca++ and Mg++ were omitted.

C¹⁴O₂ was collected in a KOH center well and converted to BaC¹⁴O₃ for assay of radioactivity. At the end of incubation the slices were drained of buffer and homogenized for 3 minutes in 50 ml 1:1 acetone-ethanol in a Waring blender.* After centrifugation the supernatant was taken to dryness on a steam bath under nitrogen, and the dried residue saponified by autoclaving 4 hours at 15 pounds with 5 ml 3 N KOH. Then 5 ml absolute ethanol were added and the non-saponifiable lipid extracted with three 30-ml aliquots of n-heptane. This was back-washed with 1 N KOH and water. Aliquots of the concentrated heptane extract were dried on steel planchets and counted directly to determine *non-saponifiable lipid radioactivity*. In several experiments

* We are indebted to Dr. Joseph H. Bragdon for this simple procedure which he has found to give essentially complete extraction of liver lipids.

cholesterol radioactivity was measured by preparing and counting the digitonide. Results were essentially the same when obtained by direct counting of heptane extracts. On the basis of this correlation, the incorporation into *non-saponifiable lipid* fraction may be taken as a measure of cholesterol synthesis. The KOH phase, after extraction of cholesterol was acidified and the alcohol and volatile fatty acids removed by heating on a steam bath. Non-volatile fatty acids were extracted with three 30-ml aliquots of n-heptane and back-washed with 10% acetic acid and water. This treatment, as shown by zero time control studies, effectively removed all traces of the original labeled acetate. After concentration, aliquots of this extract were plated and counted directly (*non-volatile fatty acid radioactivity*). Acetoacetate was decarboxylated at end of incubation by addition of aniline citrate to the acidified medium(4). The $C^{14}O_2$ liberated, representing carboxyl-carbon, was collected in a KOH center well and assayed as for metabolic $C^{14}O_2$. Inhibitors[†] were made to the appropriate final concentrations in buffered medium as sodium salts. All radioassays were carried out using the windowless flow proportional counter of Robinson(5). Self-absorption corrections on $BaCO_3$ and cholesterol digitonide samples were made to the 3.1 mg/cm² point.

Results. *In vitro studies with alpha-phenyl-n-butyrate.* At 1×10^{-3} M alpha-phenyl-butyrate strongly inhibited incorporation of sodium acetate-1- C^{14} into cholesterol (*non-saponifiable lipid*). In the typical experiment summarized in Table I the rate of incorporation in presence of the drug was only about one-half that of control. Extent of inhibition did not appear to be a function of the time of incubation. However, it did vary from rat to rat. In 6 experiments with incubation times ranging from $\frac{1}{2}$ to 2 hours, inhibition varied

TABLE I. Effect of Alpha-phenylbutyrate on Acetate-1- C^{14} Incorporation into Lipids.*

Flask	Conc. of alpha-phenyl-butyrate	Total radioactivity in lipid fractions (10^4 c.p.m./g liver slices)	
		Non-saponifiable lipid	Non-volatile fatty acids
1	0	1.06	.093
2	0	1.00	.088
3	1×10^{-3} M	.48	.030
4	"	.61	.040

* 30 min. incubation, 400-420 mg liver slices in 3.5 ml medium. 1μ c sodium acetate-1- C^{14} added (1 mc/mM).

from 32% to 62%, mean 42%. The reason for this variation, as well as for variation in rate of incorporation in normal control rats noted by many laboratories, is not entirely understood(6-8).

Incorporation into non-volatile fatty acids was also inhibited to about the same extent. In 5 experiments it ranged from 23% to 62% with a mean of 41%.

The observed inhibition of both cholesterol and fatty acid synthesis from acetate suggested that the site of action of the drug might be at an early step common to both biosynthetic pathways. Incorporation of labeled acetate into acetoacetate was measured and, as shown in Table II, was also inhibited by about 50%.

At 1×10^{-3} M the drug did not significantly affect the endogenous oxygen consumption of liver slices (Table II), suggesting that the effects on lipid synthesis are not due to a basic disruption of the over-all tissue metabolism. This is further indicated for, in a number of experiments the oxidation of labeled acetate to $C^{14}O_2$ was decreased only by 5-15% (see Table II). Apparently the reactions of tricar-

TABLE II. Effects of Alpha-phenylbutyrate on Oxygen Uptake, Acetate-1- C^{14} Oxidation and Incorporation into Acetoacetate.*

Phenylbutyrate concentration	Total radioactivity (10^4 c.p.m./g liver slices)		Oxygen uptake (μ l/g wet wt)
	Acetoacetate	CO_2	
0	.80	2.34	927
1×10^{-3} M	.41	2.06	896

* 120 min. incubation, 430-480 mg liver slices in 3.0 ml medium. 0.1μ c sodium acetate-1- C^{14} added (1 mc/mM).

[†] Alpha-phenyl-n-butyric acid was purchased from Distillation Products Industries; α,α' -diphenylbutyrate and β,β' -diphenyl- α -ethylpropionate were synthesized in Dr. Evan C. Horning's laboratory; α,α' -diphenylvalerate and 1-dimethylamino-2,2 -diphenylpentane HCl were made by Smith, Kline and French Laboratories (SKF 2314 and SKF 3019 A).

TABLE III. Effects of Compounds Structurally Related to Alpha-phenylbutyrate on Acetate-1-C¹⁴ Oxidation and Incorporation into Non-saponifiable Lipid Fraction.

Inhibitor	Total radioactivity (10 ⁴ c.p.m./g liver slices)			
	CO ₂		Non-saponifiable lipid	
	Control	Inhibited	Control	Inhibited
α, α' -diphenylbutyrate (1×10^{-3} M)*	21.0	20.0	1.02	.36
β, β' -diphenyl- α -ethyl propionate (1×10^{-3} M)*	21.0	16.6	1.02	.44
α, α' -diphenylvalerate (1×10^{-3} M)†	24.3	12.2	.92	.17
<i>Idem</i> (1×10^{-3} M)‡	14.4	10.1	.031	.025

* 120 min. incubation. 500 mg liver slices in 5 ml medium. 1 μ c (= 1 μ M) acetate-1-C¹⁴ added.

† 60 min. incubation. 380-400 mg liver slices in 3.5 ml medium. 1 μ c acetate-1-C¹⁴ added.

‡ 60 min. incubation. 400-410 mg liver slices in 3.5 ml medium. 150 μ M unlabeled acetate with 1 μ c (= 1 μ M) of acetate-1-C¹⁴.

boxylic acid cycle and reactions of terminal oxidation can proceed with little or no impairment in the presence of these concentrations.

Inhibition of C¹⁴O₂ production, while on the average considerably less than inhibition of lipid synthesis (mean of 7 experiments 15%), was nevertheless highly significant. In 2 experiments this inhibition rose to about 40%. This finding, as discussed further below, suggests that there is inhibition either in activation of acetate(9) or in condensation reaction through which acetyl-coenzyme A (acetyl CoA) enters the tricarboxylic acid cycle(10). When the concentration of alpha-phenylbutyrate was raised to 1×10^{-2} M, incorporation of labeled acetate into cholesterol and fatty acids was almost completely inhibited and incorporation into acetoacetate was inhibited by more than 70%. However, at this concentration there was also inhibition of oxygen consumption, as much as 25% in some experiments, and inhibition of the rate of incorporation of alanine-1-C¹⁴ into liver proteins(11). At this higher concentration, then, the drug interfered with tissue respiration and presumably also with energy metabolism.

In vivo studies with alpha-phenyl-n-butyrate. It has been proposed(3) that alpha-phenyl-butyrate might interfere with cholesterol synthesis by forming an extremely stable CoA complex, thus reducing the effective levels of the coenzyme. The concentrations of CoA in livers of rats fed alpha-phenylbutyrate, 200 mg/kg/day for 4 weeks, were measured (12). The values found for control animals were: 163, 228, 179 units/g; for the drug-fed

animals, 203, 207, 226 units/g.† While the results in such a small group cannot be considered conclusive they suggest that any dramatic effects on levels of available CoA are not to be expected. Since the experiments of Klein and Lipmann(13) show that liver CoA must fall to 50 or 60 units/g before lipid synthesis is inhibited it is unlikely that availability of CoA is a limiting factor in lipid synthesis of phenylbutyrate-fed rats. Total liver cholesterol in these drug-fed animals also remained at normal levels.

In vitro studies with related compounds. Several compounds structurally related to alpha-phenylbutyrate have been studied *in vitro* in a similar manner. As shown in Table III, 2 of these (α, α' -diphenylbutyrate and β, β' -diphenyl- α -ethyl-propionate) have similar effects. At 1×10^{-3} M they inhibit cholesterol synthesis by somewhat over 50% without markedly inhibiting acetate oxidation. The third compound (SKF 2314 = α, α' -diphenylvalerate) is a considerably more potent inhibitor of cholesterol synthesis, effective even at concentrations of 1×10^{-5} M. These compounds, aromatic acids with structures close to that of alpha-phenylbutyrate, have been previously shown by Brodie *et al.*(14) and by Cook *et al.*(15) to inhibit a number of reactions in biotransformation of drugs. The spectrum of reactions inhibited is quite broad (*e.g.* hydroxylation, barbiturate side-chain oxidation, dealkylation) but they share in common a requirement for oxygen and triphos-

† We wish to thank Dr. Earl R. Stadtman for his assistance in the CoA analyses.

phopyridine nucleotide and localization in microsomes(13).

Studies by La Du *et al.*(16) have shown further that the carboxyl group is not an absolute structural requirement for inhibition in microsomal systems. 1-dimethylamino- 2,2-diphenylpentane hydrochloride (SKF 3019 A) proved extremely potent in inhibiting the dealkylation reaction. It was of interest, then, to test this compound in our system. At 1×10^{-3} M it inhibited acetate incorporation into cholesterol by 53% and acetate oxidation to CO_2 by 17%.

Discussion. These data suggest that the effect of alpha-phenylbutyrate on acetate incorporation into lipids represents inhibition at one of the earliest levels of acetate metabolism, probably before formation of aceto-acetate. The site of inhibition could be either: 1) in the formation of acetyl-CoA; or 2) in subsequent acetylation reactions involving acetyl-CoA; or 3) both.

The fact that incorporation into lipids was generally inhibited more strongly than oxidation to CO_2 does not rule out inhibition at the activation step. Since the reaction pathway to CO_2 is favored (Table III), the percentage inhibition in this direction, when the supply of acetyl-CoA is decreased, might well be less than the percentage inhibition in less favored pathway toward cholesterol and fatty acid synthesis.

Under 2) above are included key reactions of acetylation of oxalacetate to form citrate and acetylation of acetyl-CoA to form acetoacetyl-CoA. A differential inhibition of these 2 acetylation reactions would account for the observation that CO_2 formation was inhibited less than incorporation into lipids. Inhibition of acetoacetate formation by ethylthioacetate and benzoate has been demonstrated by Avigan, Quastel, and Scholefield(17). They showed that these compounds probably exert their inhibitory effects as CoA derivatives. In a similar manner alpha-phenylbutyrate may inhibit only after formation of an acyl-CoA complex.

Whether *in vitro* inhibition of cholesterol synthesis from acetate described here represents the mechanism for *in vivo* effects on

serum cholesterol levels reported by Cottet *et al.*(1-3) is not indicated by these studies and is the subject of further investigation.

The activity in our system of the drug potentiators studied by Brodie *et al.* is provocative. While the compounds may be acting in different ways in the 2 systems, further studies are called for to determine whether the manifold effects on drug metabolism may be mediated through effects related to the effects of alpha-phenylbutyrate on acetate metabolism.

Summary. 1) Alpha-phenyl-n-butyrate at 1×10^{-3} M has been shown to decrease rate of incorporation of acetate-1- C^{14} into cholesterol and into fatty acids by rat liver slices. 2) Incorporation of acetate into aceto-acetate is inhibited about the same extent, while oxidation to CO_2 is generally less strongly inhibited. 3) Endogenous oxygen consumption can proceed relatively unimpaired in the presence of 1×10^{-3} M alpha-phenylbutyrate. 4) Several compounds structurally related to alpha-phenylbutyrate are also demonstrated to inhibit acetate incorporation into lipids. 5) The possible relation between present observations on inhibition of lipid synthesis and studies of Brodie *et al.*(14) on drug potentiators is discussed.

1. Cottet, J., Redel, J., Krumm-Heller, C., and Tricaud, M. E., *Bull. Acad. Nat. de Med.*, 1953, No. 25-26-27, 441.

2. Bargeton, D., Krumm-Heller, C., and Tricaud, M. E., *C. R. Soc. de Biol.*, 1954, No. 1-2, 63.

3. Cottet, J., Mathivat, A., and Redel, J., *La Presse Medicale*, 1954, v62, 939.

4. Edson, N. L., *Biochem. J.*, 1935, v29, 2082.

5. Robinson, C. V., *Science*, 1950, v112, 198.

6. Fredrickson, D. S., Loud, A. V., Hinkelman, B. T., Schneider, H. S., and Frantz, I. D., *J. Exp. Med.*, 1954, v99, 43.

7. Tomkins, G. M., and Chaikoff, I. L., *J. Biol. Chem.*, 1952, v196, 569.

8. Langdon, R. G., and Bloch, K., *ibid.*, 1953, v202, 77.

9. Jones, M. E., Lipmann, F., Hilz, H., and Lynen, F., *J. Am. Chem. Soc.*, 1953, v75, 3285.

10. Stern, J. R., and Ochoa, S., *J. Biol. Chem.*, 1951, v191, 161.

11. Anfinsen, C. B., and Steinberg, D., *ibid.*, 1951, v189, 739.

12. Stadtman, E. R., Novelli, G. D., and Lipmann,

F., *ibid.*, 1951, v191, 365.

13. Klein, H. P., and Lipmann, F., *ibid.*, 1953, v203, 101.

14. Brodie, B. B., Axelrod, J., Cooper, J. R., Gaudette, L., La Du, B. N., Mitoma, C., and Udenfriend, S., *Science*, 1955, v121, 603.

15. Cook, L., Macko, E., and Fellows, E. J., *J.*

Pharm. and Exp. Therap., 1954, v112, 382.

16. La Du, B. N., Horning, E. C., Wood, H. B., Trousof, N., and Brodie, B. B., *Fed. Proc.*, 1954, v13, 377.

17. Avigan, J., Quastel, J. H., and Scholefield, P. G., *Biochem. J.*, 1955, v60, 329.

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A Method for Quantitating Flexibility Changes of Pelvic Joints in Rats and Mice.* (21993)

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An increase in flexibility of the pelvic joints in mice occurs during pregnancy and following appropriate hormonal treatment(1-3). This increase was determined by manual manipulation of the pelvis. A more reliable method, suitable for both the mouse and rat, was devised to measure small differences which can be statistically analyzed.

Materials and methods. To quantitate changes in flexibility of the sacroiliac joints the animal is sacrificed, skinned, its body divided at the level of the iliac crests, and the tail severed at the 3rd caudal vertebra. The muscles, which arise in part from the dorsal aspect of the sacrum and 1st caudal vertebra to insert on the extremities, are severed at their origins and reflected laterally. The extremities and pelvic viscera are then removed. All of the musculature attached to the innominate bones is severed close to the bone, except the iliac origins of the sacrospinalis, the iliococcygei (abductor caudae internus), and the pubococcygei (abductor caudae externus; Fig. 1, PC) which are left intact. (Careless removal of the iliac origin of the sacrospinalis might damage the dorsal ligaments of the sacroiliac joint (Fig. 1, SI); the latter 2 muscles hold the ischial tuberosities in a convenient position for making subsequent measurements. Preliminary tests showed that

the weight used to flex the sacroiliac joint produced maximum flexion whether or not these muscles were present.) A straight dissecting needle is inserted into the neural canal as far as it can go under moderate pressure (Fig. 1, C). The free end of the needle is slipped into an adjustable clamp which holds the specimen in a horizontal position 2 inches from a transparent plastic protractor (Fig. 1, B, J, M). The specimen's position is adjusted so that the zero line of the protractor is aligned with the dorsal-most portion of the left iliac crest and ischial tuberosity (Fig. 1, F'). To assure constant, correct alignment the positioning of the specimen is performed while viewing it through a hole (4 mm in diameter) in the center of an opaque rectangle (3 x 5 inches) placed 5 inches from the protractor (Fig. 1, A, L). A 50 g weight sufficient to produce a 3°-5° displacement of the ischial tuberosity in normal rats or a 2 g weight sufficient to produce a 1° displacement in normal mice is suspended from the symphysis pubis (Fig. 1, K). A raising of the protractor to realign the zero line with the dorsal-most portion of the iliac crest is necessary at this time because the crest is slightly elevated as the ischial tuberosity is lowered by the weight. While viewed through the hole in the rectangle a movable transparent plastic arm attached to the protractor is lowered by the observer's finger so that a black line running through the length of the arm aligns with the dorsal-most portion of the now displaced ischial tuberosity (Fig. 1, I, G'). Displace-

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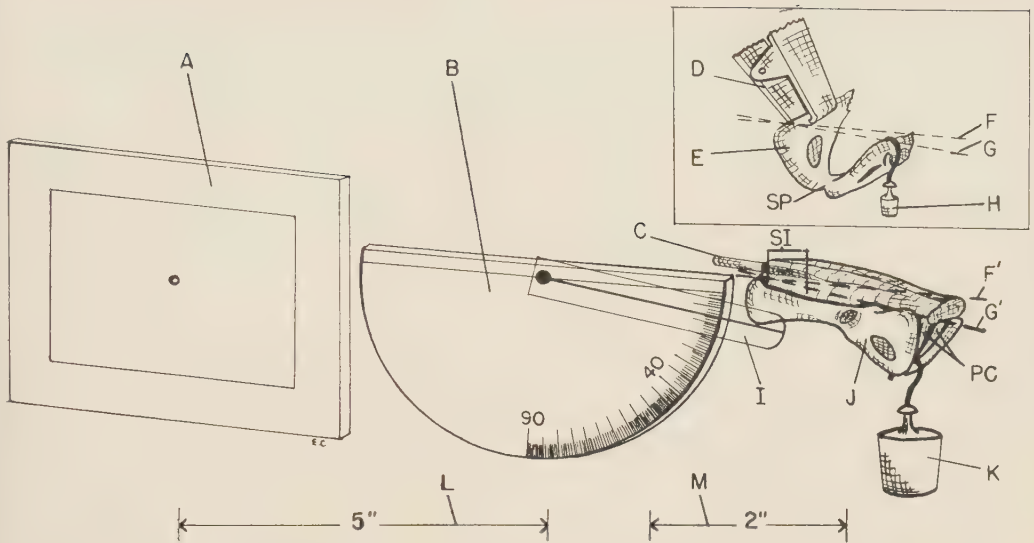


FIG. 1. Diagram of apparatus used for quantitating flexibility changes of pelvic joints in rats and mice. Relative dimensions of rat's pelvis are shown.

ment was measured in degrees at 30 seconds after the weight was applied by recording the position of the line of the arm in relation to the degree markings on the protractor.

To quantitate changes in flexibility of the symphysis pubis of the mouse (Fig. 1, SP)

(interpubic synchondrosis in the rat) the innominate bones are separated from the vertebral column by transecting the ilia close to the acetabula. The left ischium is placed in an adjustable clamp which holds the innominates in a horizontal position (Figs. 1, E; 2).

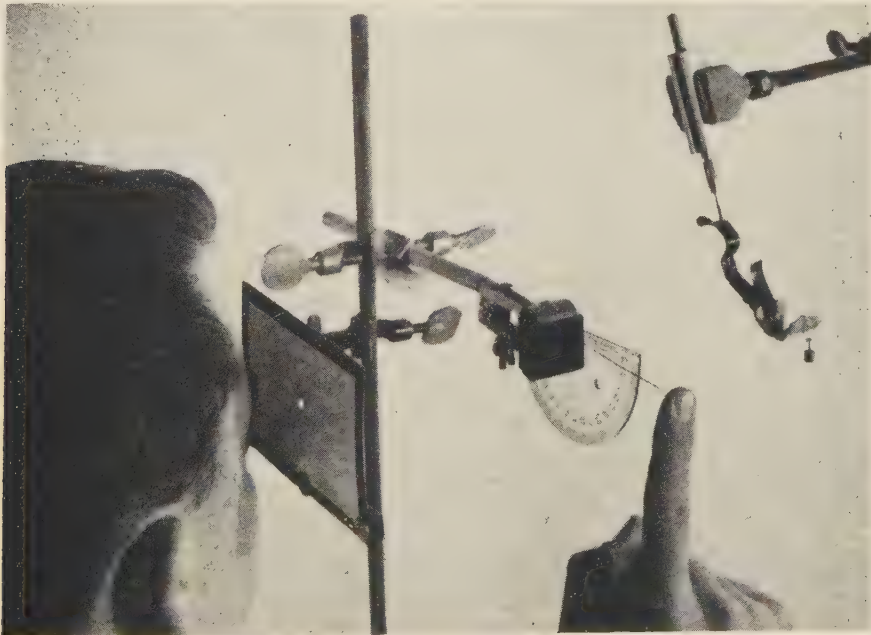


FIG. 2. Photograph of apparatus being used to quantitate flexibility of a rat's interpubic synchondrosis.

Each ischial tuberosity is 2 inches from the protractor. While viewed through the hole in the rectangle the zero line of the protractor is aligned with the dorsal-most portions of both ischial tuberosities (Fig. 1, F). A weight sufficient to produce a 1° displacement in normal animals (1 g for rat, 0.1 g for mouse) is suspended from the right ischium midway between the tuberosity and the acetabulum (Fig. 1, H). Fifteen seconds from the time the weight was applied the amount of displacement of the right ischial tuberosity is recorded using the arm attached to the protractor in the same manner as described for determining sacroiliac joint flexibility (Fig. 1, G). Once adept, one can perform the entire procedure in less than 10 minutes from the time the animal is sacrificed.

Detailed reports in which this method was used to quantitate changes in pelvic joint flexibility in the rat and mouse during pregnancy and following estrogen and relaxin treatment are forthcoming.

Summary. A method was devised for measuring small differences in pelvic joint flexibility in rats and mice. The procedure in which weights are used to produce joint flexion and a protractor to measure the amount of flexion in degrees is described.

1. Gardner, W. U., *Am. J. Anat.*, 1936, v59, 459.
2. Crelin, E. S., *ibid.*, 1954, v95, 47.
3. ———, *PROC. SOC. EXP. BIOL. AND MED.*, 1954, v86, 22.

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Response of Mice to Standard Infecting Doses of *Mycobacterium tuberculosis* var. *hominis*.* (21994)

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We described(1) a procedure which could be used to determine the effectiveness of chemotherapeutic agents for the suppression of an experimental tuberculous infection of mice. Data available at that time indicated that the method was highly reproducible. This method now has been applied to the *in vivo* assessment of chemotherapeutic activity for a period of 5 years. Not only has the original estimation of the reliability of the method for this purpose been confirmed, but, in addition, data has been collected during this period which is of importance in regard to the response of untreated normal mice to tuberculous infection. One to 6 groups of untreated normal mice have been infected almost every month during this period. The nature of the response of these untreated control mice to inoculation with a standard infecting dose of *M. tuberculosis* var. *hominis* H37Rv will be the subject of the present communication.

Method. Since the standardized mouse infection procedure has already been described in detail(1) it will suffice here to state that "Strong A" strain mice weighing between 18 and 22 g were maintained and housed under standard conditions and infected intraven-

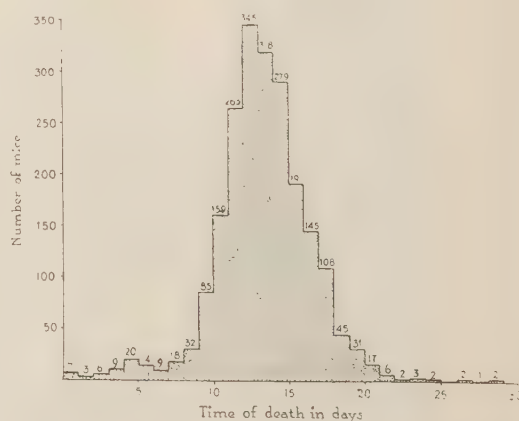


FIG. 1. Distribution of deaths of mice infected with 1.0 mg of H37Rv strain of *M. tuberculosis* var. *hominis*.

* This study was aided in part by a grant from Parke, Davis & Co., Detroit, Mich.

TABLE I. Median Survival Times of Groups of Strong A Strain Mice Injected Intravenously with 1.0 mg of the H37Rv Strain of *M. tuberculosis* var. *hominis*.

Year	Month												Yearly means
	Jan.	Feb.	Mar.	Apr.	May	June	July	Aug.	Sept.	Oct.	Nov.	Dec.	
1950		12.5	13.5	15.0	15.0	14.0	13.0			13.0	13.0	13.5	13.57
		13.0	14.5	16.0	14.0	14.0	13.5			12.5		11.5	
			14.0		14.0	13.5				11.5			
			15.0			12.5							
1951	12.5	12.0		14.5	15.5	11.5	14.5	15.0	12.5	14.5	12.5	14.0	13.53
	14.0	12.5		14.0	13.5	11.5		16.0	15.0	14.0	12.5		
	14.0					11.0		16.0	12.0	13.0	11.5		
						13.5				14.5	13.0		
1952										15.5			13.11
	16.0	13.0	12.0	12.0	11.0	11.5	12.5	13.0	14.5	13.0	15.0	13.5	
	14.0	14.5	12.5	14.5	12.5	14.5	14.5	10.0	14.5	14.0	12.5	10.5	
	14.0	13.5	11.0	12.5	12.0	13.5			10.5	11.5	11.5	15.5	
1953										12.5	13.5	14.5	12.70
	15.5	13.0	11.5	14.5	12.5	12.5	13.0	12.5	13.0	13.5	13.0	14.5	
	11.5	12.5	14.5	12.0	12.0	11.5	12.0		13.0	14.0	14.5	13.5	
	14.0	12.0		12.5	14.5	8.0	10.5		12.5	12.0		12.0	
1954										14.5	12.5		13.47
										11.5	11.5		
										15.0	19.5	14.0	
										12.0		14.0	
Monthly means	14.0	13.10	13.21	13.40	13.21	12.53	12.88	13.05	13.13	13.43	13.11	13.25	

ously with 1.0 mg wet weight of the virulent H37Rv strain of *Mycobacterium tuberculosis* var. *hominis*. Following inoculation a record was made of the time of death in days of each mouse. Each mouse was autopsied shortly after death in order to confirm that death was due to tuberculosis. The cumulative percent of mice dead was plotted against time of death on logarithmic probability paper, the median survival time estimated and the 95% confidence limits calculated using the method of Litchfield(2).

Results. Fig. 1 shows the distribution of deaths of 2,123 of the 2,173 untreated normal mice contained in the 180 groups which were infected over the 5-year period 1950 through 1954. The mean survival time of the mice shown in Fig. 1 was 13.84 ± 3.16 days. Fig. 1 does not include 4 mice which died at the time of inoculation; 37 mice which were accidentally killed, and 9 mice which lived more than 30 days. Inclusion of the 9 mice which lived longer than 30 days gives a mean survival time of 14.02 ± 4.56 days.

Table I shows the distribution of median survival times of the 180 groups of mice according to both year and month for the years 1950 to 1954 inclusive. The yearly means of the median survival times varied between 12.70 and 13.57 days. Analysis of variance showed that statistically these yearly means did not differ significantly. The means of the monthly median survival times varied between 12.53 and 14.0 days. Again, analysis of variance revealed that statistically these means did not differ significantly.

Discussion. The preceding data clearly show that with standardization of technic and by the use of a strain of *Mycobacterium tuberculosis* var. *hominis* maintained at constant virulence(3) the response of an inbred strain of mice to tuberculous infection can be remarkably uniform. The demonstration of such uniformity of response and the definition of the conditions necessary to produce it are highly desirable preliminaries to attempts to measure, on the one hand, the degree of natural or acquired immunity to tuberculosis or,

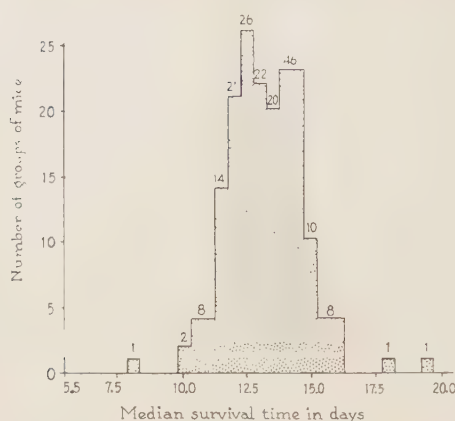


FIG. 2. Distribution of median survival times of groups of mice injected with 1.0 mg of H37Rv strain of *M. tuberculosis* var. *hominis*.

on the other, the virulence of strains of *Mycobacteria*.

The data also offer no support for the occurrence of any yearly or seasonal variation in the resistance of this strain of mice to tuberculous infection. This might in part be a reflection of the fact that these mice were housed in an air conditioned animal room and were therefore not subjected to extreme variations in temperature. However, this room

faces south and during the summer temperatures in this room ranging between 90° and 95°F have been recorded. The observed extreme temperatures in this room have been approximately 65°F in the winter and approximately 95°F in the summer.

Summary. 1. Over a 5-year period, 1950 through 1954, one to 6 groups per month of 10 to 30 mice each were infected intravenously with a 1 mg inoculum of the H37Rv strain of *Mycobacterium tuberculosis* var. *hominis*. 2. The median survival times of 177 (98.3%) of the 180 groups infected during this period fell between the 10th and 16th day inclusive. Statistical analyses revealed that there was no significant difference between the yearly means of the median survival times nor was there significant difference between the monthly means of the median survival times.

1. Youmans, G. P., and Youmans, A. S., *Am. Rev. Tuberc.*, 1951, v64, 541.

2. Litchfield, J. T., Jr., *J. Pharmacol. and Exp. Therap.*, 1949, v97, 399.

3. Youmans, G. P., *Annals N. Y. Acad. Sci.*, 1949, v52, 662.

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Hypothalamic Lesions in Goldthiogluucose Injected Mice.* (21995)

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(Introduced by F. J. Stare.)

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Recent investigations have demonstrated the essential similarity of goldthiogluucose obesity and hypothalamic obesity in mice (1-3). This in turn led to the re-examination of the possibility that hypothalamic lesions may be involved in the mechanism of action of

goldthiogluucose despite the fact that other investigators (4,5) did not succeed in demonstrating such lesions. Since the possibility existed that through a process of reabsorption and repair, any damage to the central nervous system may be at least in part obscured in the obese animal during the period required for the obesity to develop, it was decided to examine injected animals shortly after goldthiogluucose administration. Goldthiomalate, though similar to goldthiogluucose in gold content, molecular weight and toxicity does not produce obesity (6), consequently it was de-

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[†] Life Insurance Medical Research Fund Predoctoral Fellow.

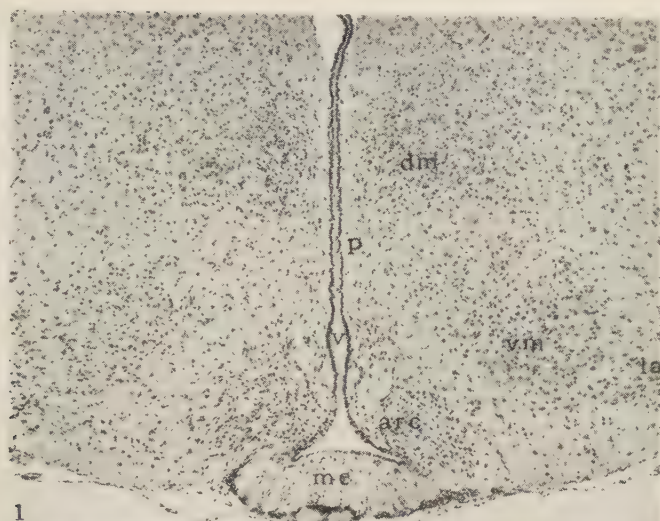


PLATE I. Photomicrographs ($\times 65$) taken of hematoxylin and eosin stained sections through tuberal or infundibular region of the hypothalamus showing the same area in 3 different animals.

FIG. 1. Section through hypothalamus of a goldthiomalate treated mouse, indicating a normal hypothalamic nuclear configuration and containing a normal number of neurons. Photomicrograph is labeled to indicate normal landmarks and nuclei seen in this region of the hypothalamus: dm, dorsomedial nucleus; f, fornix; p, periventricular region; v, third ventricle; vm, ventromedial nucleus; lat, lateral hypothalamic area; arc, arcuate nucleus; me, median eminence.

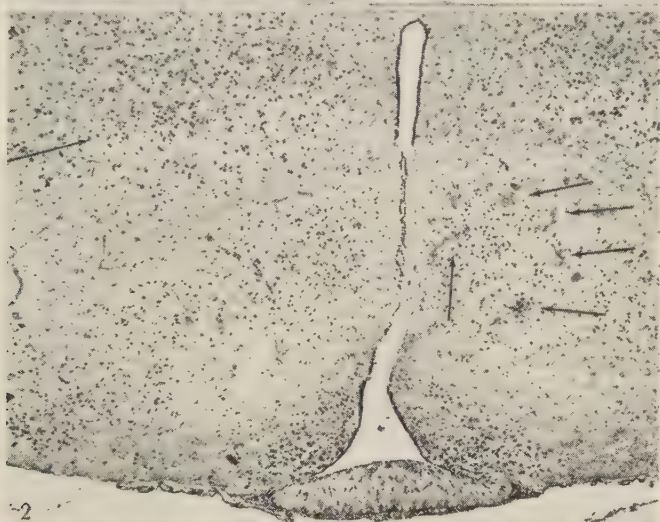


FIG. 2. Same area as in Fig. 1 of hypothalamus of mouse three days after the administration of goldthioglucose. Note marked loss of neurons and presence of pyknotic cells in ventral part of hypothalamus. Normal hypothalamic nuclear pattern has been lost. Arrows on right side of photomicrograph point to punctate hemorrhages. Arrow on left points to line of demarcation between area of edema (ventral) and normal tissue (dorsal).



FIG. 3. Section of hypothalamus of obese mouse three months after the administration of goldthioglucose. Compare with Fig. 1 and 2. This section is slightly posterior to Fig. 1 (more ventral position of fornix (f)). Although there is no evidence of edema as in Fig. 2, there is an obvious decrease in the number of cells. The ventromedial nucleus (vm) can barely be made out as a discrete nuclear mass. The tissue forming the median eminence (bottom) has been destroyed.

cided to use goldthiomalate injected animals as controls.

Materials and methods. All the animals used were female Swiss mice averaging in age from 4 to 6 months. Out of 100 or more animals of each type, the following representative animals were examined: 6 uninjected control mice, 7 mice injected with goldthiogluco- 24 to 72 hours prior to autopsy, 5 mice which had become obese as a result of goldthiogluco- 24 to 72 hours prior to autopsy, 4 mice which failed to become obese after being injected with goldthiogluco- at least 3 months prior to autopsy, and finally 5 mice injected with goldthiomalate 24 to 72 hours prior to autopsy. The non-obese mice weighed 25 to 35 g; the obese mice weighed 45 to 65 g. Each of the treated mice was injected with 1 mg/g of body weight of the appropriate gold compound. This dosage represents a 50% lethal dose(6). Each brain was carefully dissected free of its bony enclosures and fixed for 24 hours in 10% neutral formalin. The overlying thalamus, subthalamus, and cerebral cortex were not trimmed and were used for control neural tissue in each specimen. The blocks of brain were dehydrated in a routine manner, embedded in paraffin and serial sectioned at 10 μ throughout the entire block. The sections were stained with hematoxylin and eosin and examined.

Results. Hypothalami of the goldthiogluco- injected animals showed remarkable lesions within one to 3 days generally consisting of diffuse and punctate hemorrhages, widespread and marked edema involving more neural tissue than the hemorrhagic areas and pyknosis and degeneration of nerve cells throughout areas of edema and hemorrhage (Fig. 2, 4, 5, 7). The ventral portion of the third ventricle, especially the infundibular recess, also showed changes in that it was frequently engorged with blood and the lining ependymal cells were sometimes pyknotic and disrupted (Fig. 2, 4, 7). Although the anatomical extent and severity of the hypothalamic damage varied from animal to animal, it was clear in all cases that at least the ventral portion of the hypothalamus was involved. In the most severe instances, there was marked destruction of tissue at the base of the brain

between the optic chiasm and the mamillary bodies, so that the neural tissue lying ventral and lateral to the third ventricle was fragmented and necrosed. In these specimens the usual landmarks in the ventral hypothalamus such as the supra-optic nucleus, ventromedial nucleus, median eminence, arcuate nucleus, and the lateral hypothalamic area were completely destroyed. In the less severe cases, the hypothalamic tissue at the base of the brain was intact, and the hypothalamic nuclei recognizable as clumps of cells, but the neurones in this area showed the signs of damage, and the parenchyma was edematous and hemorrhagic (Fig. 2, 4).

The limits of this edemous hypothalamic tissue were in all cases sharply demarkated (Fig. 2, 4, 5, 7). Dorsal to the edematous area, the neurones appeared normal. There was an abrupt transition from the normal tissue to the less eosinophilic edematous tissue that contained damaged neurones. The punctate and diffuse hemorrhagic areas were nearly always found within the edematous regions. They were most frequently found in the neural tissue adjacent to the ventral portion of the third ventricle and in the lateral hypothalamic area (Fig. 2, 4, 7).

Anteriorly, at the level of the optic chiasm, the damage appeared restricted to the most ventral nuclei adjacent to the chiasm. A bit posteriorly, the damage was more extensive, reaching more dorsally, so that in several specimens the paraventricular nucleus was involved, but at the same level, the laterally placed fornix was unaffected in all specimens. The supra-optic nucleus, the suprachiasmatic nucleus, and the ventral parts of the anterior and lateral hypothalamic areas were damaged in all specimens.

In the middle hypothalamic region (infundibular), the most extensive and severe damage was found. The ventromedial nucleus, the ventral part of the lateral hypothalamic area, the arcuate nucleus, and the median eminence were always destroyed (Fig. 2, 4, 5, 7). In 3 animals the dorsomedial nucleus and the perifornical area were also involved (Fig. 3, 5), but in no cases were regions lateral or dorsal to these damaged. In the posterior hypothalamus, only the mamillary bodies and

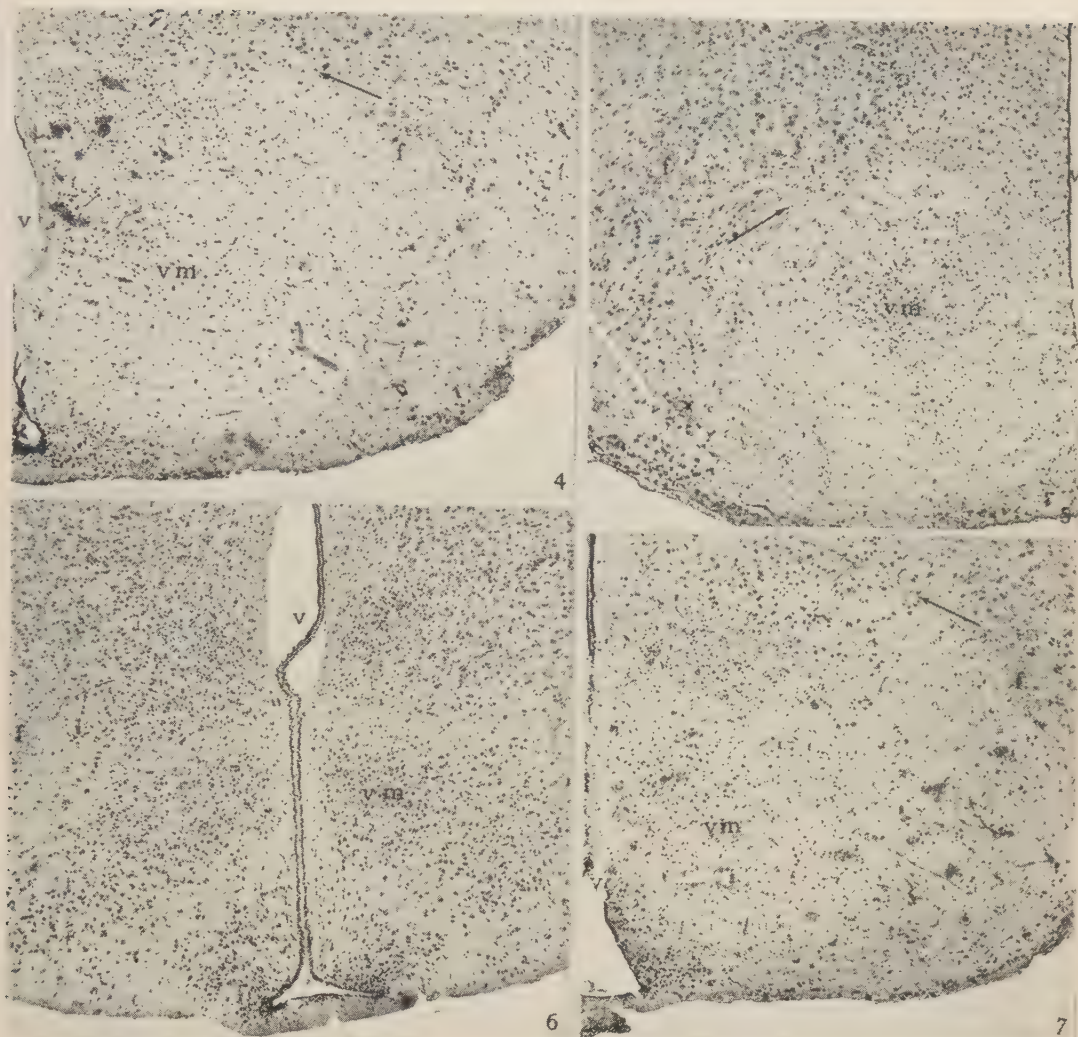


PLATE II: Photomicrographs ($\times 65$) taken of hematoxylin and eosin stained sections through tuberal or infundibular region of hypothalamus showing the same area in 4 different mice. Fig. 4, 5, and 7 were goldthioglucose treated. Fig. 6 was goldthiomalate treated. In Fig. 4, 5, and 7 note paucity of neurons in ventral hypothalamus, the pyknotic cell in edematous regions, and in Fig. 4 and 7, the widespread hemorrhages. In each figure position of the ventricle (v), ventromedial nucleus (vm), and fornix (f) is labeled. Arrows point to line of demarcation between destroyed (ventral) and normal (dorsal) regions.

the lateral area showed damage; the Fields of Forel and the cerebral peduncle outside the hypothalamus appeared normal.

Some inflammatory cells (polymorphonuclear leukocytes and lymphocytes) were present in the damaged areas, but their number was not great. Neurophagia and glial proliferations were observed, but these were scanty. In fact the large loss of neurons could not be accounted for in the 3 days after goldthioglucose administration by the amount of

neurophagia observed. Presumably some of the neurons were lost by disruption and dissolution. There were no other areas of hemorrhage, cellular degeneration, and edema in the remaining parts of the brain overlying the hypothalamus.

The only lesion found in the brains of obese mice successfully injected with goldthioglucose at least 3 months prior to investigation was a paucity of neurons in the hypothalamus especially the ventral portion. This finding is

clearly demonstrable by a comparison of Fig. 1 and 3. The extent of the loss of neurons and replacement of glia was variable in the specimens examined. In one case this resulted in loss of discrete hypothalamic nuclear pattern which can be seen in the normal mice. In other specimens the nuclei could be recognized as clumps of cells, but they contained fewer neurons (Fig. 3).

Sections of the brain of animals given goldthiomalate showed nothing of the characteristic damage found in Swiss mice given goldthioglucoase. In fact, as concerns the hypothalamus in particular, there were no differences from those of uninjected control mice. However, 2 of these animals had hemorrhages in the lateral ventricles, especially the frontal poles.

Examination of sections of brain of mice injected with goldthioglucoase at least 3 months prior to autopsy and which failed to become obese revealed no distinct abnormality. The hypothalamic nuclei appeared distinct, and the number of neurons appeared within normal limits, though in some there seemed to be fewer neurons in a strip of hypothalamus lying ventral to the third ventricle.

The evidence presented here seems to support the conclusion that goldthioglucoase produces obesity through causing hypothalamic lesions, always involving the ventromedial nuclei. Damage to the ventromedial nuclei has been shown to be essential to the production of obesity in rats(7) and mice(2). It is remarkable that, even though the hypothalamic area involved is often very large, only hyperphagia appears to result. The inactivity shown by hypothalamic mice(2) is not present in goldthioglucoase obesity(6). Goldthioglucoase obese mice do not show gonadal atrophy by weight(8).

Discussion. It would appear that the presence of the glucose component in the molecule makes this limited hypothalamic portion of the brain barrier much more permeable to this compound than to compounds

not containing glucose. The hypothalamic area containing the feeding centers has been previously postulated to contain "glucoreceptors"(7). Larsson has since shown that this feeding center area effectively concentrates radioglucoase with the receptivity apparently characteristic of the area rather than of discrete units(9). The results here would support the idea that the ventromedial feeding center area is a "glucoreceptive" area, in the sense postulated in the glucostatic theory of the regulation of food intake.

Summary. Goldthioglucoase injections, in the dose which produces obesity in mice, cause in these animals extensive hypothalamic damage involving the ventromedial nuclei. Such lesions are permanent in animals which in fact become obese, but are not apparent in animals unsuccessfully injected. Goldthiomalate, though as toxic as goldthioglucoase, does not cause obesity; its injection does not lead to hypothalamic lesions. Implications of these findings as regards the mechanism of the regulation of food intake are discussed.

The authors gratefully acknowledge the assistance of Dr. Charles DeWan. The goldthioglucoase used in these studies as well as in previous studies was obtained through the courtesy of Dr. Edward Henderson and the Schering Corp., Bloomfield, N. J.

1. Bates, M. W., Zomzely, C., and Mayer, J., *Am. J. Physiol.*, 1955, v181, 187.
2. Mayer, J., French, R. G., Zighera, C. Y., and Barnett, R. J., *ibid.*, 1955, v182, 75.
3. Mayer, J., and Zighera, C. Y., *Experientia*, 1955, v11, 358.
4. Brecher, G., and Waxler, S. H., *PROC. SOC. EXP. BIOL. AND MED.*, 1949, v70, 489.
5. Owen, J. A., Jr., Parson, W., and Crispell, K. R., *Metabolism*, 1953, v2, 362, quoting unpublished investigations of Haymaker, W.
6. Marshall, N. B., and Mayer, J., *Am. J. Physiol.*, 1954, v178, 271.
7. Mayer, J., *Physiol. Rev.*, 1953, v33, 472.
8. Marshall, N. B., and Mayer, J., to be published.
9. Forssberg, A., and Larsson, S., *Acta Physiol. Scand.*, 1954, v32, suppl. 115 (Part 2).

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Prevention of Cobalt-Induced Polycythemia in Rats by Calcium Ethylene Diamine Tetra Acetic Acid. (21996)

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Polycythemia results from oral or parenteral administration of cobalt(1-5). It is known that this hematopoietic action of cobalt can be prevented by simultaneous feeding of sodium salt of ethylene diamine tetra acetic acid(6,7) and it is probable that under these circumstances cobalt is chelated in the gastro-intestinal tract preventing its absorption. As EDTA administered either as sodium or calcium salt rapidly combines with cobalt to form non-ionizable complexes, it is believed that administration of either of these compounds by the same route as cobalt does not effectively measure prevention of a polycythemic response to cobalt(8,9). It was of interest in this study to determine if EDTA administered as calcium salt prevents production of polycythemia when administered by a different parenteral route than cobalt.

Materials and methods. Six normal female rats of the Long-Evans strain, weighing 225 to 270 g, received daily intraperitoneal injections of 0.5 ml of a 1% saline solution containing 0.125 mg of cobalt as cobaltous sulfate. The injected solution also contained 0.200 mg of iron as ferric chloride to prevent iron shortage due to increased synthesis of hemoglobin under the influence of cobalt(10). Another group of 6 rats received daily 50.0 mg of CaEDTA subcutaneously in addition to the intraperitoneally administered cobaltous sulfate and ferric chloride. Red cell counts,

hemoglobin, and hematocrit determinations were made after 34 days, and injections were continued for a total of 74 days, at which time red cell volumes were also determined. Six rats were retained without treatment to serve as controls. Blood samples were withdrawn under ether anesthesia from the external jugular vein into heparinized syringes. Hemoglobin concentrations were determined by the Turner method(11). Hematocrits were determined by Van Allen or Wintrobe tubes after centrifugation at 3,000 r.p.m. for 30 minutes. Blood volumes were determined by the Fe^{59} -labeled red cell method(12).

Results. Rats receiving cobalt showed significant increases in red cell count, hematocrit, hemoglobin, and total red cell volume, whereas no increase was found in rats injected with both cobalt and CaEDTA (Table I).

This experiment confirms the effectiveness of the amounts of cobalt and iron previously reported as capable of producing polycythemia in the rat(13). CaEDTA even when administered by a different parenteral route than cobalt still prevented the development of polycythemia by the cobalt.

Preliminary experiments, in which radioactive cobalt (Co^{60}) was used to determine the rate of excretion, showed that cobalt was excreted at a more rapid rate when CaEDTA was administered simultaneously.

Summary. Cobalt injected intraperiton-

TABLE I. Haematological Data in Rats Injected with Cobalt, Alone or in Combination with CaEDTA. Six rats in each series.

Treatment	Days inj.	Body wt, g	Hemoglobin, g/100 ml	Hemoglobin, g/100 g body wt	Hematocrit, %	Red blood cells $\times 10^6/\text{mm}^3$	Red blood cell vol/100 g body wt, ml
Cobalt	0	252	—	—	—	$7.6 \pm .3$	—
	34	255	$16.9 \pm .8$	—	64.3 ± 4.9	$10.7 \pm .6$	—
	74	278	$16.0 \pm .5$	$.831 \pm .033$	63.0 ± 4.1	—	$3.25 \pm .20$
Cobalt + CaEDTA	0	248	—	—	—	$7.6 \pm .1$	—
	34	258	$14.7 \pm .7$	—	47.6 ± 1.7	$8.2 \pm .4$	—
	74	273	$13.9 \pm .3$	$.632 \pm .018$	50.0 ± 1.6	—	$2.27 \pm .09$
Control	34	225	$15.0 \pm .1$	—	$48.8 \pm .8$	$8.4 \pm .4$	—
	74	276	$13.0 \pm .5$	$.592 \pm .027$	48.0 ± 1.1	—	$2.25 \pm .09$

eally into rats receiving calcium ethylene diamine tetra acetic acid subcutaneously did not produce the usual polycythemic response.

The author expresses his appreciation to A. N. Contopoulos of the Institute of Experimental Biology for advice and assistance.

1. Waltner, K., *Arch. Exp. Path. Pharmacol.*, 1929, v141, 123.
2. Waltner, K., and Waltner, K., *Klin. Wochsch.*, 1929, v8, 313.
3. Berlin, N. I., *J. Biol. Chem.*, 1950, v187, 41.
4. Garcia, J. F., Van Dyke, D. C., and Berlin, N. I., *PROC. SOC. EXP. BIOL. AND MED.*, 1952, v80, 472.
5. Orten, J. M., Underhill, F. A., Mugrage, E. R., and Lewis, R. C., *ibid.*, 1931, v29, 174.

6. Child, G., and Leonard, P., *Fed. Proc.*, 1951, v10, 286.
7. Child, G., *Science*, 1951, v114, 466.
8. Foreman, H., Vier, M., and Magee, M., *J. Biol. Chem.*, 1953, v203, 1045.
9. Martell, A. E., personal communication.
10. Van Dyke, D. C., Asling, C. W., Berlin, N. I., and Harrison, R. G., *PROC. SOC. EXP. BIOL. AND MED.*, 1955, v88, 488.
11. Turner, A., *Bull. U. S. Army Med. Dept.*, 1946, v5, 605.
12. Berlin, N. I., Huff, R. L., Van Dyke, D. C., and Hennessy, T. G., *PROC. SOC. EXP. BIOL. AND MED.*, 1949, v71, 176.
13. Berlin, N. I., Ph.D. Thesis, Univ. Calif., June, 1949.

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Total Cardiac By-pass Utilizing Continuous Perfusion from Reservoir of Oxygenated Blood.* (21997)

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The goal of our cardiac research efforts has been the development of simple, safe, and effective technics for direct vision intracardiac surgical procedures. The demonstration that total cardiac by-pass with right ventricular cardiectomy and septal suture can be successfully performed even in seriously ill patients by utilizing controlled cross circulation (1-4) doubtless will stimulate further rapid developments in this field.

This experimental study has been carried out on dogs to evaluate a method of substituting arterial and venous reservoirs of blood for the donor in the cross circulation set-up. By such a plan, it would be possible to perform intracardiac operations in a dry heart without the need of a donor in the operating room. Such a scheme immediately poses problems of

procurement and storage of blood to supply the arterial reservoir. In this present experimental study intended to develop a feasible technic for total cardiac by-pass utilizing the reservoir method of perfusion, the arterial blood was obtained prior to the intended perfusion from a donor used as an oxygenator entirely independent of the perfusion set-up. The use of arterialized venous blood to supply the arterial reservoir has been described elsewhere (5).

Method. Collection. Ordinary 1,000 cc plasma vac[§] bottles were used as reservoirs. These containers were readied for use by instilling in each 100 cc 5% dextrose and water solution containing heparin (25 to 32 mg) and at the same time exhausting the vacuum completely. Arterial blood was collected into these bottles (while in the inverted position) by gravity using a commercially available plastic collection set^{||} connected to a plastic cannula^{||}

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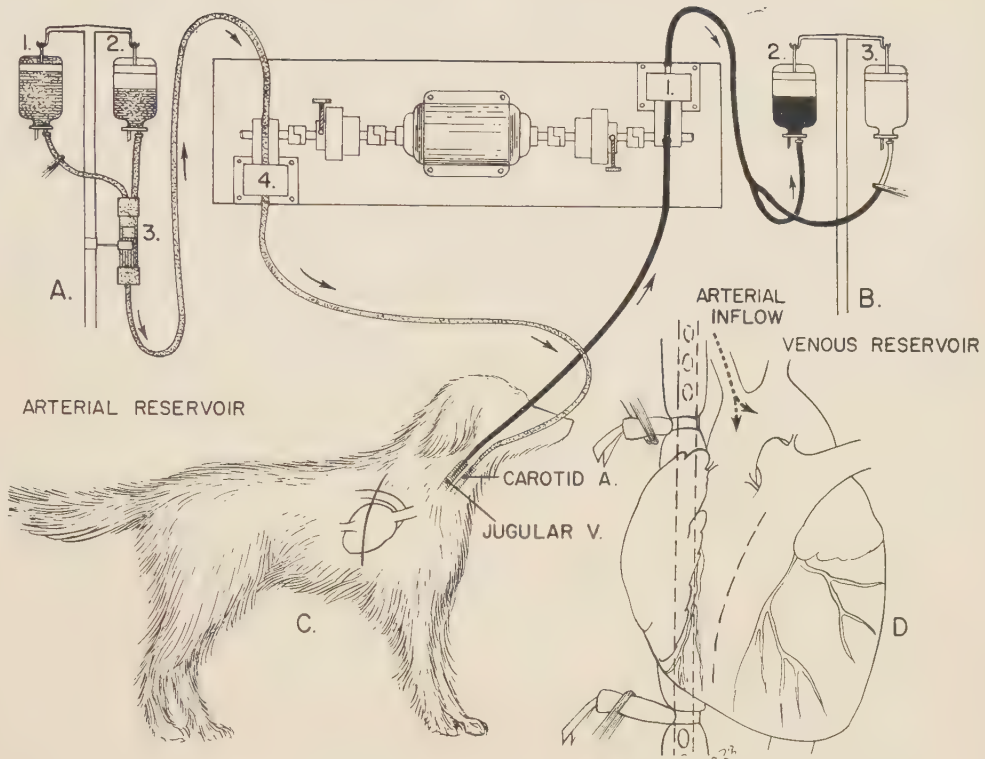


FIG. 1. Arterial reservoir perfusion. A. Arterial reservoir showing bottles of arterial blood (1 & 2), blood filter (3), and arterial pump (4). B. Venous portion of perfusion circuit with venous pump (1) and bottles (2 & 3) for collection of venous blood withdrawn from the patient's caval system. C. Recipient's relationship to the system is depicted in center of diagram and site of cannulations of right carotid artery and jugular vein are shown. Arterial cannula is inserted 3-4 cm down the carotid artery and ligated in place. Venous cannula with holes at tip and along shaft (the distance between these 2 sets of holes is determined by the space between the vena caval occluding tapes of the animal concerned) is passed down the jugular vein into the venae cavae until the holes are in the proper relation to the occluding tapes as indicated in D. At top center is pumping assembly which consists of separate pumps at each end driven by a single motor (center) and separate speed changers which are interposed between the motor and each pump head.

that had been placed in the donor's femoral artery. This cannulation in the donor animal was ordinarily done under pentothal sodium anesthesia or curare paralysis to permit endotracheal intubation for artificial respiration with 100% oxygen during the period of collection. However, in an occasional trained or cooperative animal cannulation was possible with only local procaine anesthesia. Synchronously with the withdrawal of arterial blood from the donor animal stored venous blood was infused into the donor's femoral vein by means of a cannula similar to that in the artery. To avoid any undesirable alterations in the donor's blood volume this venous

infusion was maintained at a rate equal to the removal of arterial blood. The quantity of arterial blood withdrawn from the donor animal was dictated by the desired rate and duration of perfusion and usually ranged between 2000 to 3500 cc. After collection, the arterial blood was ordinarily placed in a water bath maintained at 39°C where it remained until needed for the perfusion. However, when there was to be a longer time interval between collection and perfusion the blood was kept refrigerated (at 4°C). In such instances it was rewarmed to 39°C before it was used for perfusion. *Operative method.* Mongrel dogs weighing from 4.4 to 11.5 kg were used as re-

recipient animals. All were premedicated with morphine 3-4 mg/kg of body weight and atropine gr. 1-100, and then anesthetized with 2½% solution of pentothal sodium injected intravenously. Endotracheal tubes were inserted, and except for the period of perfusion the animals were artificially respired throughout the operative procedure. Details of the methods of arterial and venous cannulations, vena cava occlusion, connections to the pump† are depicted in Fig. 1 and were identical in every respect to those of the recipient animals in the cross circulation study group reported in detail elsewhere(1). The rates of perfusion were calculated on the basis of the reduced flow principle(6) and ranged between 25 to 30 cc/kg of recipient body weight/min. The length of perfusion was arbitrarily set at 20 minutes, during which time total cardiac and pulmonary by-pass was in effect. *Reservoir perfusion* (Fig. 1). As indicated above, the patient's (or recipient's) relationship to the extracorporeal circuit (C & D) is unchanged from that employed for cross circulation. The donor, on the other hand, has been replaced by containers of arterial blood (A 1 & 2) and empty venous receptacles (B 2 & 3) respectively. The 2 bottles of arterial blood were connected by means of a Y to a blood filter (A3) similar to those used for infusions of blood on the hospital wards. Blood was allowed to fill the filter system with the latter inverted so that all air was displaced from the chamber. The outflow of the filter chamber was then connected to the plastic tubing leading to the arterial pump (A4). After these connections were made, the pump was turned on for a few moments to completely fill the arterial circuit before the arterial outflow limb of the pump was connected to the arterial cannula (C & D) of the recipient. With this done, the arterial circuit was complete and after the recipient's caval cannula was connected to the tubing leading to the venous pump (B) perfusion was begun. Blood was withdrawn from the recipient's vena cavae via the cannula (C & D) connected to the plastic tubing by the venous pump (B1) and was collected into either venous receptacle

TABLE I. Changes in Donor Hemoglobin and Platelets.*

Hemoglobin	
Control	13.6 g %
Final	12.8
Platelets	
Control	512,000
Final	365,000

* Avg figures for 10 donors, 3000 cc arterial blood was withdrawn from the femoral artery of each synchronously with infusion of equal amount of venous blood into femoral vein over a 1-2 hr period.

(B2 or 3). In practice the arterial pump drew blood from only one bottle at a time and was replaced by another full one so that at all times there was an uninterrupted perfusion. Similarly when the venous reservoir (B2) was full, the venous outflow was diverted into the empty reservoir (B3) and (B2) was replaced by another empty bottle. The output from each pump was preset before the perfusion at the rate desired. Usually for the study animals these were set equal. However, occasionally when blood loss from a cardiectomy was anticipated an imbalance was intentionally created by reducing the venous pump rate below that of the arterial.

Observations. A total of 34 animals was used in this study (17 as recipients and 17 as oxygenators) to evaluate and develop this method of reservoir perfusion. Certain physiologic determinations were made on both the recipient and blood donor animals in an attempt to assess the alterations imposed by the method.

Hemoglobin and platelet alteration in the donor. Blood samples were taken from the donor's femoral vein cannula for hemoglobin and platelet determinations just before and after the collection of the arterial blood. The changes in 10 donors are tabulated in Table I. In these animals 3,000 cc of arterial blood was withdrawn from the femoral arteries while a like amount of citrated dog blood was administered into the femoral vein over a period of approximately one to 2 hours. The infused blood had been obtained from several dogs at sacrifice or as the by-product of a previous perfusion and had been stored for periods up to 5 days. Despite the varying quality and age of this infused venous blood the altera-

† Sigmamotor Inc., Middleport, N. Y.

TABLE II. Hematologic and Metabolic Changes* Produced in Recipient after Reservoir Perfusion for 20 Min.

Recipient	pH	O ₂ (vol %)	CO ₂ (vol %)	Hgb. (g)	Platelets per mm ³
Control					
Artery	7.48	15.33	34.16	—	—
Vein	—	10.93	41.94	11.2	317428
Final					
Artery	7.26	18.53	30.67	—	—
Vein	—	12.03	38.96	13.4	182000

* The above are mean figures for 8 animals. The apparently low control arterial O₂ content is consistent with the avg control hemoglobin level (see text).

tions noted were relatively insignificant. In most of the donors the hemoglobin drop was approximately one-third of their normal level.

Changes in the recipients. In Table II are summarized the results of pH, O₂, CO₂, hemoglobin and platelet determinations made on 8 of the recipients just before and immediately after reservoir perfusion during which total cardiac by-pass was in effect. The most pronounced effect noted was the reduction in pH which in this instance fell from an average preperfusion level of 7.48 to 7.26 immediately postperfusion. This acidosis was similar to the observations made in total cardiac by-pass by other methods in studies previously reported(1,7,8) and probably was also due to the accumulation of acid metabolites during the perfusion period. The blood oxygen and carbon dioxide content determinations showed very little change. On first inspection the average control arterial O₂ content would appear to be low. However, this figure is consistent with the low control hemoglobin levels. The finding of an anemia in new stock animals admitted to the colony has not been unusual especially during the winter months. The rise in hemoglobin and its resultant increase in arterial O₂ content at the end of the perfusion was comparable to the hemoconcentration noted in previous perfusion experiments(7,8). The average blood platelet count fell to approximately one-half of its control level. The greatest drop in platelets demonstrated by any of the recipient animals was one-third of the control figure and ordinarily the recovery was relatively prompt so that the postoperative count was usually doubled by the following

day and was back to its control level within 2 to 3 days.

There was no donor mortality in this study. During the early phases before the optimum heparin dosage had been established postoperative bleeding in the recipients was a frequent complication, and 6 of the first 7 recipients succumbed to this complication. However, of the last 10 recipients who received blood collected in less heparin (25-32 mg/liter) this complication has not occurred and 8 were long term survivors. The 2 deaths resulted from avoidable errors; one from avulsion of a carotid artery during decannulation and one from pulmonary edema caused by the inadvertent rapid infusion of a large amount of saline in the early postoperative phase.

Discussion. The results herein reported indicate that the use of the described method of continuous arterial perfusion from a reservoir of oxygenated blood is a feasible means for totally by-passing the heart and lungs for a time interval sufficient to perform intracardiac operations under direct vision. By the removal of the donor from the operating theater the treatment of patients with intracardiac abnormalities would be further simplified by a reduction in the number of personnel and the amount of equipment necessary. This method of collection of arterial blood from the donor with synchronous replacement by an equal quantity of compatible venous blood into the same body region would appear to be a simple and safe procedure for the donor associated with about the same risk as a conventional blood transfusion. If the blood is collected within a short time of its intended use the recipient (or patient) would appear to derive almost the same physiological benefits as when the donor is interposed directly into the extracorporeal circulation.

An obvious limiting factor inherent in this reservoir type of perfusion for clinical use is the fact that the length of the perfusion is limited by the amount of arterial blood collected for the reservoir. In practice several factors would appear to minimize this objection. First, clinical experience has confirmed the safety and practicability of total body perfusions at normal temperature utilizing re-

duced flow rates (25 to 40 cc of blood/kg body weight/minute). Secondly, experience gained in the corrective surgical treatment of intracardiac defects by the use of controlled cross circulation has indicated that, with direct vision, the majority of ventricular defects required well under 20 minutes of intracardiac operating time. These two factors coupled with the obvious fact that many of the urgent candidates for intracardiac operations are infants or small children make the technic herein described attractive for further investigation and clinical application.

In the present study the donor animal was used as an oxygenator for the procurement of arterial blood with which to supply the arterial reservoir. It is interesting to note that there was a relatively small depreciation in the donor's hemoglobin and platelets even though he "processed" a volume of blood which was usually greater than his total blood volume over a relatively short period of time. Also, this occurred in spite of the somewhat dubious quality of the stored venous blood infused into the donor dogs. This observation attests to the efficacy of the dog's hematopoietic regenerative powers and it would be reasonable to anticipate even less depreciation in these factors clinically with the use of a better quality of bank blood for the replacement.

Experimentally it was usually necessary to anesthetize the donor animal for the collection of arterial blood, but this would not be required in the clinical application of the method. Under these conditions, the collection could be performed via an arterial puncture of several compatible donors or from a single donor using local procaine infiltration of the puncture sites.

Summary. 1. A simple method permitting total by-pass of the heart and lungs for the performance of intracardiac surgical operations is described. 2. This method utilizes

continuous perfusion of the recipient's (patient's) arterial system from a reservoir of oxygenated blood. An equivalent quantity of venous blood is removed from the recipient's superior and inferior cavae. 3. A pump is utilized to control this exchange between the recipient and the arterial and venous reservoirs. 4. Arterial blood for the reservoir was obtained from a donor animal used as an oxygenator independent of the perfusion system. This was done by collecting arterial blood from the donor's femoral artery into a heparin glucose solution while stored venous blood was infused into his femoral vein at an equal rate. Ordinarily 2,000 to 3,500 cc of arterial blood was collected from a single donor dog in this fashion. 5. The optimum dose of heparin to prevent clotting in the arterial blood collected for this purpose was 25 to 32 mg/liter of blood. 6. The simplifications of the clinical application of this method are briefly discussed.

1. Warden, H. E., Cohen, M., Read, R. C., and Lillehei, C. W., *J. Thor. Surg.*, 1954, v28, 331.
2. Lillehei, C. W., *Postgrad. Med.*, 1955, v17, 388.
3. Lillehei, C. W., Cohen, M., Warden, H. E., and Varco, R. L., *Surgery*, 1955, v38, 11.
4. Lillehei, C. W., Cohen, M., Warden, H. E., Ziegler, N., and Varco, R. L., *Surg., Gynec., and Obstet.*, Oct., 1955.
5. Warden, H. E., Read, R. C., DeWall, R. A., Aust, J. B., Cohen, M., Ziegler, N., Varco, R. L., and Lillehei, C. W., *J. Thor. Surg.*, in press.
6. Cohen, M., and Lillehei, C. W., *Surg., Gynec., and Obstet.*, 1954, v98, 225.
7. Cohen, M., Warden, H. E., and Lillehei, C. W., *ibid.*, 1954, v98, 523.
8. Warden, H. W., Cohen, M., DeWall, R. A., Schultz, E. A., Buckley, J. J., Read, R. C., and Lillehei, C. W., *The Surgical Forum, American College of Surgeons*, W. B. Saunders Co., Philadelphia, 1954, pp. 22-28.

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Effect of Sex Hormones on Conversion of Folic Acid to *Citrovorum* Factor by Rat.* (21998)

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The stimulatory influence of folic acid (PGA) and an inhibitory effect of aminopterin on the tissue growth response to estrogen in the female chick and rat was first reported by Hertz and Tullner(1). Gelfant *et al.*(2) reported that citrovorum factor (CF) partially reverses the inhibitory effect of aminopterin on estrogen induced mitoses in the rat uterus. Aminopterin is reported(3) to inhibit the urinary excretion of CF by rats receiving PGA.

The present report concerns the study of the effect of sex hormones on the *in vivo* conversion of PGA to CF by male rats.

Methods. Adult male rats of the Sprague-Dawley strain were placed individually in metabolism cages equipped with metal collecting funnels. Rats were maintained on Purina laboratory chow (Ralston Purina Co.) starting at the weaning stage and throughout the experimental period. Three separate experiments were conducted. In Exp. 1, 9 rats were randomized into 3 groups of 3 rats each. Group 1 received the Purina laboratory chow unsupplemented. Groups 2 and 3 received a daily supplement of 100 γ PGA by stomach tube. In addition to the latter, Group 3 received intramuscular injections of 7.7 γ estradiol dipropionate in sesame oil. In Exp. 2, 19 rats were randomized into 6 groups, 5 of which consisted of 3 rats each while Group 4 comprised 4 rats. Group 1 received the basal diet unsupplemented. Groups 2, 3 and 4 received a daily supplement of 100 γ PGA by stomach tube. In addition to PGA, Groups 3 and 4 received daily intramuscular injections of 0.5 mg testosterone and 10 γ estradiol di-

propionate respectively. Groups 5 and 6 were controls for groups 3 and 4 and received daily intramuscular injections of 0.5 mg testosterone and 10 γ estradiol dipropionate respectively. In Exp. 3, 19 rats were randomized into 5 groups, 4 of which consisted of 4 rats each with the exception of Group 1 which had 3 rats. Group 1 received the basal diet unsupplemented. Groups 2, 3, 4 and 5 received a daily supplement of 100 γ PGA by stomach tube. In addition to PGA, Group 3 received daily intramuscular injections of 0.5 mg testosterone, Group 4 received daily intramuscular injections of 10 γ estradiol dipropionate, while Group 5 received daily injections of same amounts of both the sex hormones. Urine collections were started 24 hours after the rats had been maintained on the respective diets and were continued daily for a one to 2-week test period. The 24-hour urine collections from the rats of the same group were combined and an aliquot of the latter was filtered, neutralized and autoclaved for 30 minutes at 120°C. The heated samples were cooled, reneutralized, diluted and assayed. The CF content of the urine samples was determined by using the Bacto-Cf Assay Medium (Difco Laboratories). *Leuconostoc citrovorum* ATCC 8081† was used as the test organism, and Leucovorin (Lederle) was employed as the standard. The cultures were incubated 48 hours at 37°C and growth was measured by turbidimetric procedure. Since leucovorin has been reported to be half as active as the CF isolated from a horse liver(4), the microbiological assay values were divided by 2 in order to express the results in terms of

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† A recent report (Felton, E. A., and Niven, C. F., *J. Bact.*, 1953, v65, 482) concerning a taxonomic study on the culture *Leuconostoc citrovorum* ATCC 8081 suggests that the latter organism is a typical strain of *Pediococcus cerevisiae* as described earlier by Pederson (*Bact. Rev.*, 1949, v13, 225).

TABLE I. Effect of Sex Hormones on Urinary Excretion of Citrovorum Factor by Rats Receiving PGA.

Exp.	No. of rats	Supplement* to basal/day	Urinary excretion of CF, mg/rat/day	
			1st wk	2nd wk
1	3	0	135	
	3	PGA, 100 γ	750	
	3	<i>Idem</i> + EDP,† 7.7 γ	1125	
2	3	0	155	
	3	PGA, 100 γ	785	
	3	<i>Idem</i> + Test.,‡ .5 mg	810	
	4	" + EDP, 10 γ	1320	
	3	Test., .5 mg	75	
	3	EDP, 10 γ	230	
3	3	0	232	170
	4	PGA, 100 γ	902	1068
	4	<i>Idem</i> + Test., .5 mg	1015	960
	4	" + EDP, 10 γ	1320	1454
	4	" + Test., .5 mg + EDP, 10 γ	1518	1495

* PGA administered by stomach tube while sex hormones were given by intramuscular injections.

† EDP = Estradiol dipropionate in sesame oil.

‡ Test. = Aqueous suspension of testosterone.

the naturally occurring CF. At the end of the test period the rats from Exp. 2 were sacrificed, accessory sex organs and the testis removed, blotted and weighed.

Results. Administration of 100 γ PGA to male rats receiving a practical diet increases 4 to 6-fold the urinary excretion of CF (Table I). This increase is enhanced 50% by daily administration of 7.7 γ or 10 γ of estradiol dipropionate. On the other hand daily administration of 0.5 mg testosterone to male rats receiving PGA does not increase the urinary excretion of CF. The stimulatory effect of estradiol dipropionate on the CF excretion is not reversed by simultaneous administration of testosterone. It is interesting to note that

rats receiving estradiol dipropionate alone also show a 50% increase in CF excretion, while the rats receiving testosterone alone show a 50% decrease in CF excretion.

It is apparent from the results in Table II that administration of 100 γ of PGA to the male rats does not influence the weight of the accessory sex organs or the testis. Estradiol dipropionate alone or together with PGA markedly decreases the size of the accessory sex organs and the testis. On the other hand testosterone alone or in combination with PGA significantly increases the size of the accessory sex organs.

Discussion. The results presented indicate clearly that *in vivo* conversion of PGA to CF, as measured by the urinary excretion of CF after administration of PGA is enhanced by estradiol dipropionate but is not influenced by administration of testosterone. It is interesting to note that the stimulatory effect of estradiol dipropionate on CF excretion is not reversed by the simultaneous administration of testosterone. Administration of 10 γ estradiol dipropionate alone gives a 50% increase in CF excretion, while the latter decreases by 50% on administration of 0.5 mg testosterone. Estradiol dipropionate markedly decreases while testosterone increases the size of the accessory sex organs. Under these conditions supplementing of PGA does not influence the size of the accessory sex organs. The administration of ascorbic acid to rats receiving PGA has been reported to enhance the urinary excretion of CF(3). Dietary homocysteine and cysteine enhance the urinary excretion of CF by rats receiving PGA

TABLE II. Effect of PGA and Sex Hormones on the Accessory Sex Organs and Testis in the Male Rat.

Supplement* to basal/day	Final body wt, g	Accessory sex organs†				Testis
		Seminal vesicle	Coagulating gland	Ventral prostate	Dorsal prostate	
0	342	108	36	131	65	444
PGA, 100 γ	361	100	45	150	68	453
<i>Idem</i> + Test., .5 mg	352	173	99	236	135	452
" + EDP, 10 γ	312	45	12	23	21	357
Test., .5 mg	354	176	75	262	153	452
EDP, 10 γ	301	51	14	21	12	320

* PGA administered by stomach tube while sex hormones were given by intramuscular inj.

† The figures denote mg of organs/100 g body wt.

(5). Extensive studies on the effects of dietary factors on the CF excretion have not been reported. A very interesting aspect of folic acid deficiency in the rat is the diminished response to estradiol induced uterine growth(1). An interference with metabolism of C^{14} labeled estrone in aminopterin-treated rats is reported by Trunnell *et al.*(6).

Summary. Administration of estradiol dipropionate alone or together with PGA enhances the urinary excretion of CF by male rats. Testosterone alone gives a 50% decrease in the CF excretion but is without significant effect when given in combination with PGA. The stimulatory effect of estradiol dipropionate can not be reversed by testoster-

one in the amounts tested.

1. Hertz, R., and Tullner, W. W., *Ann. N. Y. Acad. Sci.*, 1950, v52, 1260.
2. Gelfant, S., Meyer, R. K., and Ris, H., *J. Exp. Zool.*, 1955, v128, 219.
3. Welch, A. D., Nichol, C. A., Anker, R. M., and Boehne, J. W., *J. Pharmacol. Exp. Therap.*, 1951, v103, 403.
4. Keresztesy, J. C., and Silverman, M., *J. Am. Chem. Soc.*, 1951, v73, 5510.
5. Doctor, V. M., and Trunnell, J. B., *Proc. Soc. Exp. Biol. and Med.*, 1954, v87, 498.
6. Trunnell, J. B., Bowman, W. E., and Hildebrandt, H. E., Abstract, *J. Clin. Endocrinol. and Metab.*, 1954, v14, 796.

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Mechanism of Protective Effect of Hydrocortisone in Staphylococci Infected Adrenalectomized Mice.*† (21999)

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The reduced natural resistance of Addisonian patients as well as of adrenalectomized animals is a general phenomenon not specific for any one type of infection or intoxication. Although the mechanism by which adrenalectomy influences resistance remains relatively obscure, replacement therapy with adrenal cortical hormones has been shown to be beneficial(1,2). On the other hand, adrenal hormones may be detrimental to host resistance(3). It has been suggested, however, that the beneficial effects of cortisone therapy are to be derived only from dosages based on requirement(4,5). In this regard, Robinson, *et al.*(6), have recently pointed out that the detrimental effect of cortisone in resistance to pneumococcal infection in rats is primarily due to overdosage with the hormone, and that an optimal dosage schedule of cortisone can

significantly enhance the host resistance of either adrenalectomized or intact infected animals. Hill, *et al.*(7), have described similar findings with systemic moniliasis in adrenalectomized mice treated with cortisone. Germuth, *et al.*(8), found that cortisone-treated rabbits could successfully resist an intravenous inoculum of *Staphylococcus aureus* which was fatal to approximately 50% of the untreated controls. They noted that the bacteria rapidly disappeared from the blood after injection in either treated or untreated animals. Kleiger and Blair(9) have described the acute death of experimental animals resulting from an inoculation of toxigenic staphylococci as an *in vivo* production of toxin by the bacteria.

It has been observed in this laboratory, that a strain of staphylococci which regularly produced a rapidly fatal bacteremia in mice after intraperitoneal inoculation proved to be relatively innocuous if given by the intravenous route. However, adrenalectomy reduced resistance to an intravenous challenge with this microorganism by a factor of at

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† The authors wish to acknowledge the technical assistance of Miss Martha Chamberlin in this study, and their indebtedness to Dr. Paul S. Nicholes for the use of the high frequency oscillator.

least 1000. This deficiency in resistance produced by adrenalectomy is apparently not caused by the impairment of phagocytic or cidal activities of the reticulo-endothelial system, but rather to an increased sensitivity of the host to the noxious effects of the bacterial protoplasm. Hydrocortisone effectively protected the adrenalectomized mice from the toxic effects of an intravenous challenge of either viable staphylococci or a bacterial extract.

Materials and methods. Mice: Eight-weeks-old male mice (CBA) were used throughout this study. Bilateral adrenalectomies were performed by the dorsal approach 18 hours before challenge. These animals were fed *ad libitum* and maintained on 0.9% saline as drinking water. All control mice were sham operated. In order to evaluate the biological effect of hydrocortisone, adrenalectomized mice were used to obviate interference from endogenous adrenal cortical secretion. *Bacteria:* The organism selected for this study was a strain of *Micrococcus pyogenes* var. *aureus*, which had been isolated from a human source. The bacterial cells were prepared for inoculation by growing them for 18 hours on nutrient agar, and then were transplanted to brain-heart infusion broth for an additional three hours of growth. The cells were harvested by centrifugation and washed once, and then resuspended with 0.9% saline. The concentration of bacteria was initially adjusted to a standard turbidity in the Klett-Summerson photocolormeter, using a 420 m μ blue filter. This was later confirmed by plate counts. *Plate counts:* Determination of viable numbers of bacteria was routinely carried out by serial 10-fold dilution of the sample, of which 0.5 ml aliquots were taken for pour plating (nutrient agar). Three plates were used for each dilution per sample. The average count was obtained after the plates had incubated for 18 hours at 37°C. *Bacterial extract:* Bacteria were grown in brain-heart infusion broth for 18 hours then sedimented by centrifugation and washed 3 times with 0.85% saline. The washed preparation was sonerated in a Raytheon high frequency oscillator at 9.0 to 9.5 kilocycles for 4 hours at 13°C. The opalescent material was removed

and centrifuged overnight in the cold. The supernatant was diluted to 1:5 and centrifuged 3 more times to reduce the viable cell count to less than 4/ml. The supernatant was then treated with (NH₄)₂SO₄ and the resultant precipitate dialyzed, reprecipitated with ethanol and finally washed with acetone and dried. *Treatment and challenge:* Hydrocortisone acetate (Merck)[†] was given by the intraperitoneal route in the standard volume of 0.1 ml. The hormone was prepared as a saline suspension and given in varying dilutions 2 hours prior to challenge. The challenge inocula of bacteria or bacterial extract were contained in volumes of 0.25 ml and introduced via the tail vein 18 hours after adrenalectomy. The LD₉₀ (90% lethal dose = 86.9 \pm 3.4%) of viable bacteria for adrenalectomized mice was found to be approximately 3 to 5 million viable cells, while the LD₉₀ (90.2 \pm 4.1%) dosage of the bacterial extract was found to consist of approximately 2.5 μ g of the dried material.

Results. Experimental. Effect of route of infection on resulting bacteremia: In preliminary work it was found that the mouse was much more resistant to infection with this microorganism by the intravenous route than by the intraperitoneal. To investigate this further, mice were divided into 2 groups and injected with 5 \times 10⁸ viable bacteria contained in a volume of 0.25 ml. One group of animals received this challenge dose by the intravenous route, while the other group was inoculated intraperitoneally. At intervals of 5, 30, 60, 120, 180 and 240 minutes following injection, 3 mice from each group were lightly anesthetized and blood samples obtained by cardiac puncture with heparinized syringes. These samples were used for estimation of the existing bacteremia (plate counts). Mice not used for bleeding were kept as infected controls. All of the intraperitoneally infected controls were dead (8/8) 24 hours after injection, whereas none (0/8) of the intravenously injected controls exhibited any obvious symptoms of infection following injection with the

[†] The authors wish to thank Dr. Elmer Alpert of Merck and Co., Rahway, New Jersey for the generous supply of hydrocortisone used in these experiments.

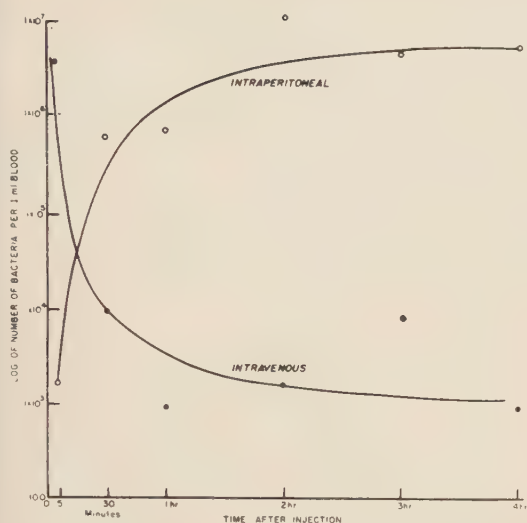


FIG. 1. Comparison of numbers of viable bacteria in blood of mice following infection by intravenous or intraperitoneal routes.

bacteria. It is evident (Fig. 1) that injection of the microorganism by the intraperitoneal route permitted the development of a rapid and overwhelming bacteremia which ultimately resulted in the death of the host. The initial bacteremia (5 minutes) undoubtedly was due only to invasion from the peritoneal cavity which was then maintained at this level by the constant infusion of bacteria produced by multiplication in the cavity. The actual extent of the invasion from the peritoneum cannot be fully appreciated unless the fate of the blood-borne bacteria is considered. The reticulo-endothelial system, which functions as a clearing mechanism for the blood, efficiently removed the inoculum dose of bacteria (Fig. 1), whereas the local response in the peritoneum is apparently overwhelmed allowing a fatal systemic invasion to occur. The two examples illustrate the extremes in bacteremia, which "represents a balance between bacterial multiplication, invasion of the blood stream, and the activity of the clearing mechanism" (10).

Bacteremia and splenic uptake of bacteria in adrenalectomized and sham operated mice: Adrenalectomy rendered the mouse highly sensitive to an intravenous inoculum of the bacteria that was relatively innocuous in the sham operated control. To investigate the influence of adrenal cortical secretion on this

aspect of resistance, adrenalectomized and sham-operated mice were inoculated intravenously with approximately 3.3×10^6 bacteria. Blood samples were obtained by intracardial puncture at the intervals noted in Fig. 2. The blood samples for each separate period were obtained from at least five mice and pooled. Likewise, the spleens of these animals were rapidly and aseptically removed, weighed, pooled and homogenized with sterile 0.85% saline in glass TenBroeck tissue grinders. Aliquots of the blood and spleen homogenates were taken for plate counting.

The inoculum used would theoretically be sufficient to produce a bacteremia of approximately 10^6 cells per ml of blood, which indicates a precipitous loss of bacteria (Fig. 2) from the blood during the initial period following injection (ca. 90% in initial 5 minutes, ca. 90% of remainder during subsequent 25 minutes). The initial high count of viable bacteria in the spleen is well correlated with the rapid clearance of the bacteria from the blood. Supplemental studies have shown that the reticulo-endothelial activity of the liver (on weight basis) was similarly effective. Since the concentration of viable bacteria in the blood rapidly fell to a relatively insignificant level, the decrease in numbers of viable

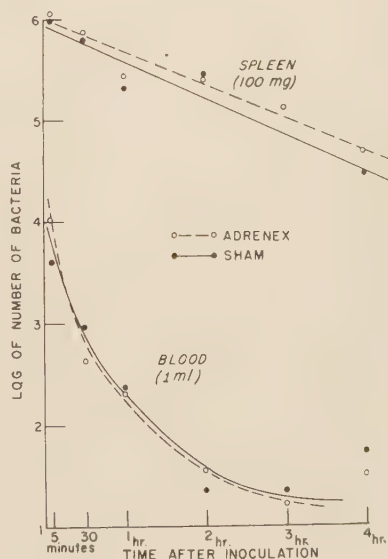


FIG. 2. Comparison of numbers of viable bacteria in blood and spleen of mice, adrenalectomized or sham-operated, following infection by intravenous route.

TABLE I. Independence of Lethal Dose on Numbers of Viable Staphylococci.

Bacterial preparation	No. viable bacteria per inoculum	Mortality	Mean survival time, min.	Probability*
A. Control	4.0×10^8	9/9	101.1	
Sonerated	6.3×10^6	10/10	85.9	P = .01
B. Control	2.0×10^8	10/10	112.6	
Sonerated	3.2×10^6	10/10	86.1	P = .01
Boiled	0.0×10^9	10/10	103.5	P = .3-.4

* Probability, using Student's "t" test, that the mean survival time of the test group is the same as the mean survival time of the control.

bacteria in the spleen was probably due to the tidal activity of the phagocytic cells. This decrease amounted to approximately 95% in 4 hours. All of the injected control sham-operated mice not used for bacteremia study survived with no evidence of symptoms, whereas all of the injected adrenalectomized mice succumbed. Despite the almost complete disappearance of bacteria from the blood and apparent destruction by the reticulo-endothelial system, symptoms of bacterial intoxication were evident in many of the adrenalectomized mice by the four hour period (ruffled fur, unsteady gait, etc.). The essential difference in the resistance of the adrenalectomized mouse when compared to its control seemed to be in its increased sensitivity to the bacteria *per se*, rather than a deficiency in ability to phagocytize and destroy the pathogen.

Toxic factor and resistance: From the results of the previous experiment it seemed possible that the digestive processes of the phagocytic cells of the reticulo-endothelial system might liberate toxic substances from the bacterial cell to the circulation which, in the adrenalectomized mouse, could attain a lethal concentration due to the greater sensitivity of this animal to noxious material. Preliminary observations showed that the lethality of various preparations of bacteria for adrenalectomized mice (measured as mean survival time) was more directly related to the concentration of bacterial substance than the numbers of viable bacteria in the inoculum. Bacteria formalinized and washed, heated to 58°C, exposed to boiling temperatures or incubated in distilled water or with antibiotics, were not less toxic than control bacterial suspensions. To simulate the result

of digestive action by phagocytic cells on the ingested microorganisms, bacteria were "solubilized" by disruption in a high frequency oscillator (sonerated) for 4 hours at 13°C.

A comparison of the toxicity of sonerated and control preparations was made following intravenous injection of the materials into adrenalectomized mice. In part A of Table I it can be seen that "solubilization" of the bacteria significantly increased the toxicity of this preparation, even though the numbers of viable bacteria were markedly reduced. The experiment was repeated (B) using a 1 to 2 dilution of the control which had been immersed in a boiling water bath for 30 minutes. Analysis of the toxicity of the control vs. the heated preparation showed no significant difference, although the latter inoculum was completely sterilized by the heat treatment. On the other hand, "solubilization" of the bacteria significantly enhanced the toxicity of the preparation. The results suggest that since the intravenously injected bacteria are rapidly localized in the tissues of the reticulo-endothelial system and therein destroyed (Fig. 2), the lethal effect of the bacteria may depend on solubilization of the ingested bacteria by the phagocytic cells and subsequent release of the toxic material to the circulation of the sensitive host.

Protective effect of hydrocortisone: The observation has been made that staphylococci administered by the i.v. route are rapidly cleared from the blood and destroyed by the cells of the R. E. system. If the digestion of these phagocytized bacteria liberates toxic bacterial substance to the circulation and thereby fatally intoxicates the adrenalectomized mouse, replacement therapy should be effective in protecting mice against a lethal

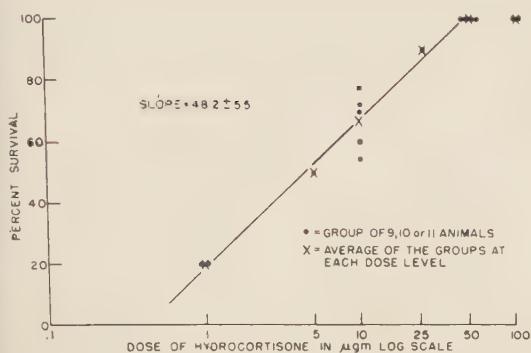


FIG. 3. Protective effect of hydrocortisone in adrenalectomized mice against 1 LD₅₀ (i.v.) of a toxic bacterial extract.

dose of either soluble bacterial extract or viable bacteria. Adrenalectomized mice were treated with graded doses of hydrocortisone two hours prior to an i.v. injection of the cell-free bacterial extract. Observations on mouse survival were routinely carried out over a period of 72 hours.

Fig. 3 illustrates the various degrees of protection, relative to hormone dosage, provided to mice by hydrocortisone against a dose of bacterial extract that was lethal to $90.2 \pm 4.1\%$ of the untreated controls (40 mice). The straight line in Fig. 3 was obtained by the method of least squares. Hormone-treated mice surviving for 24 hours following the challenge died at a rate no greater than non-challenged adrenalectomized controls.

The animals used to test the effect of hydrocortisone in mice challenged with an i.v. dose of viable staphylococci were prepared, treated and observed in essentially the same manner as in the previous experiment. In the dosage range from 0.25 to 25 γ of

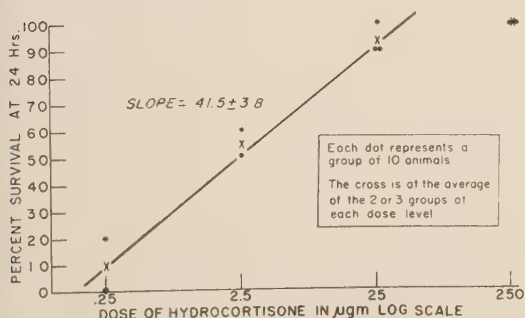


FIG. 4. Protective effect of hydrocortisone in adrenalectomized mice against 1 LD₅₀ (i.v.) of viable *M. pyogenes* var. *aureus*.

hydrocortisone, a straight line relationship of log dose to response was obtained by the method of least squares (Fig. 4). It is interesting to note that a 10-fold increase in hormone dosage did not diminish the protective response (i.e., 250 γ). Since non-moribund animals surviving 24 hours after injection died at a rate no greater than non-infected controls, it was evident that the hormone effect was not limited to a mere prolongation of survival time. Bacteremia in survivors, if present, was extremely light and the spleens contained few to no detectable organisms.

Discussion. The remarkable sensitivity of the adrenalectomized animal to noxious substances has provided a unique opportunity to study the pathogenesis of a controlled bacteremia. It has been found that after the introduction of bacteria into the blood of either adrenalectomized or sham-operated mice, the pathogens are rapidly removed from the circulation. Both the spleen and liver participated in the removal, and, on a weight basis, were similarly active. Following the uptake by the spleen, a sterilization of the bacteria ensued, and it is postulated that the digestion of the bacteria by the reticulo-endothelial cells resulted in a release of toxic (or irritating) bacterial substances. Harris and Ehrlich (11) have reported that soluble antigens of locally injected washed *Shigella* bacteria or foreign erythrocytes appear at the site of injection, draining lymph node and in the efferent lymphatics, at times which suggest solubilization and release of these antigens from the phagocytic cells of the local area. It has been found that "solubilization" (by soneration) of the bacteria decreases the survival time of i.v. injected adrenalectomized animals even though the numbers of viable bacteria are greatly reduced. Inocula of bacteria sterilized by exposure to boiling water bath for 30 minutes were as toxic as viable control material.

Since the intact mouse is markedly resistant to a given dose of either the bacteria or the bacterial extract given by the intravenous route, it is likely that the release of soluble bacterial substances by the reticulo-endothelial system in the intact mouse is accomplished without any untoward effects. However, in

the adrenalectomized mouse, which is notoriously sensitive to bacterial toxins, etc., this destruction of the bacteria does not provide suitable protection. Perhaps in the intact mouse, which has been given an intraperitoneal inoculation of the bacteria, the resulting bacteremia (Fig. 1) assumes such proportions that the R. E. system accumulation of bacterial material produced during the period of infection may be sufficient to constitute a lethal dose within a relatively short time after injection (5 to 7 hours). Smith and Keppie (12) have shown the irreversible nature of bacterial intoxication with anthrax bacillus despite control of the bacteremia by antibiotics.

Single administrations of graded doses of hydrocortisone were shown to furnish protective responses relative to hormone dosage in adrenalectomized mice injected intravenously with an LD₉₀ of either viable bacteria or bacterial extract. Since this protection did not deteriorate with time, the pathogenic activity of this organism after an intravenous injection must be essentially due to the release (solubilization) of its cellular components, rather than to multiplication and concomitant toxigenic activity. The apparent enhancement of the detoxification mechanisms of the sensitive host by hydrocortisone would require no new property not already generally ascribed to this hormone. The anti-phlogogenic activity of hydrocortisone, by limiting the chain-like sequence of events due to a noxious stimulus, can so markedly reduce the systemic reaction to the stimulus as to completely inhibit the appearance of symptoms(4).

Summary. 1. The increased tolerance of mice to an i.v. challenge of staphylococci as compared to an injection of the bacteria by the i.p. route was found to be related to the rapid clearance of bacteria from the blood following the i.v. injection. However, adren-

alectomy markedly reduced the resistance to an i.v. challenge without altering the rates of clearance or bacterial destruction by the R. E. system. 2. It was postulated that digestion of the bacteria by the R. E. system liberated bacterial material which was noxious to the adrenalectomized mouse. "Solubilization" of the bacterial preparation increased its toxicity as compared to control viable or heat-sterilized preparations, indicating the independence of this toxicity to numbers of viable bacteria. 3. Hydrocortisone pretreatment of adrenalectomized mice was found to be protective, relevant to dosage, in minute amounts, against i.v. challenge with either viable staphylococci or extracts of this organism.

1. Perla, D., and Marmorsten, J., 1941. *Natural Resistance and Clinical Medicine*, Little, Brown and Co., Boston, p475.

2. Keefer, C. S., 1954, in Lukens, F. D. W., *Medical Uses of Cortisone Including Hydrocortisone and Corticotropin*. The Blakiston Co., New York, p401.

3. Kass, E. H., and Finland, M., *Ann. Rev. Microb.*, 1953, v7, 361.

4. Dougherty, Thomas F., *Progress in Allergy*, 1954, v4, 319.

5. ———, *Adrenal Cortex*, Tr. 2nd Conf. Josiah Macy, Jr. Foundation, New York, 1950, p88.

6. Robinson, H. J., Mason, R. C., and Smith, A. L., *PROC. SOC. EXP. BIOL. AND MED.*, 1953, v84, 312.

7. Hill, D. W., Brown, H. E., and Gebhardt, L. P., *Fed. Proc.*, 1954, v13, 431.

8. Germuth, F. G., Ottinger, B., and Oyama, J., *Bull. Johns Hopkins Hosp.*, 1952, v91, 22.

9. Kleiger, B., Blair, J. E., and Hallman, F. A., *Arch. Surg.*, 1942, v45, 571.

10. Bennett, J. L., and Beeson, P. B., *Yale J. Biol. Med.*, 1954, v26, 241.

11. Harris, T. N., and Ehrich, W. E., *J. Exp. Med.*, 1946, v84, 157.

12. Smith, H., and Keppie, J., *Nature*, 1954, v173, 869.

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Bacteriostatic Properties of Some Derivatives of DDT.*† (22000)

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During an investigation of the fate of DDT in resistant insects, a number of related compounds were synthesized. Since a survey of the literature disclosed little information concerning the effects of these compounds on the metabolism of microorganisms, they were also screened for possible bacteriostatic properties.

The compounds are in general quite water insoluble and it was very difficult to obtain reproducible results using the usual turbidimetric methods to estimate microbiological growth. Such methods are also laborious, time consuming and require large amounts of compound when testing over a wide range.

For these reasons the gradient plate technique of Szybalski(16-18) was utilized for quick approximate screening.

Materials and methods. To a slanted petri plate 9 cm in diameter was added 20 ml of sterile nutrient agar (Difco) so that the bottom was just covered. After solidification the plate was leveled and an additional 20 ml of agar containing the appropriate concentration of compound to be tested was added. A waiting period of from 2 to 5 hours at 25°C proved to be sufficient to allow the compound to diffuse vertically and establish a uniform concentration slope by virtue of the thickness

TABLE I. Concentration of DDT Derivatives Required to Inhibit Growth of Microorganisms.

Compound	Source	Microorganism*				
		1	2	3	4	5
Benzophenone	†					
4,4'-Dichlorobenzophenone	13					
4,4'-Dimethoxybenzophenone	†					
4,4'-Dichloro-3,3'-dinitrobenzophenone	1					
4,4'-Dichloro-3,3'-diaminobenzophenone	12					
4-Chlorophenyl 2-thienyl ketone	4					
Phenyl 2-thienyl ketone	3				54†	
4,4'-Dimethylaminobenzophenone	†					
4-Fluoro-3-methylbenzophenone	10					
Benzhydrol	2	14	112			
4,4'-Dichlorobenzhydrol	11		112	140	42	
4,4'-Dimethoxybenzhydrol	14					
4,4'-Dichloro-3,3'-dinitrobenzhydrol	10	14	70		14	14
4,4'-Dichloro-3,3'-diaminobenzhydrol	10					
4-Chlorophenyl 2-thienylmethanol	10	70	112	70	42	42
Phenyl 2-thienylmethanol	9		132			
4,4'-Dimethylaminobenzhydrol	8				42	14
α -Methylbenzhydrol	2					
1,1-bis(p-Chlorophenyl) ethanol	5		42	42	42	
1,1-bis(p-Methoxyphenyl) ethanol	6					
1-Phenyl-1(2-thienyl) ethanol	10	224	70			
1-(4-Fluoro-3-methylphenyl)-1-phenylethanol	10		42	14	14	
1,1-bis(4-Chloro-3,5-dinitrophenyl) ethane	7		112	14	14	112
bis(p-Chlorophenyl) acetic acid	15				14	14
4-Fluoro-3-methylbenzhydrol	10	14	14		14	14

* (1) *E. coli* (sucrose negative); (2) *M. pyogenes* HSR9674; (3) *S. faecalis* ATTC1170; (4) *S. ellipsoideus*; (5) *A. suboxydans* ATTC621.

† Eastman Kodak Co.

‡ Values are given in $\mu\text{g/ml}$ required to inhibit growth. Where no value is given it is taken to mean that growth of the microorganism was not affected by the highest concentration used.

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† Data taken from a thesis submitted in partial fulfillment of requirements for the Ph.D., Department

of Chemistry, Oregon State College, June 1953.

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ratio of the agar layers. The plates were then inoculated with a thin streak of organisms using a small sterile brush. The plates were inverted and incubated for 18 hours. At this time the growth of the organisms could easily be seen and the point where growth was just inhibited determined. Concentrations up to 250 μg per ml were run at least in duplicate. The microorganisms were obtained from stock cultures belonging to the Department of Bacteriology, Oregon State College. The values obtained by this method compared within 15% of those found by standard dilution techniques when using phenol or penicillin G, sodium salt.

Results. In Table I is summarized the results obtained by screening the miscellaneous compounds for their abilities to inhibit growth of the five selected microorganisms. In general the ketones were inactive at a concentration of 250 μg per ml which was the highest level used. Many of the alcohols, however, were quite active in inhibiting growth, some at rather low concentrations. In addition to the organisms listed, most of the compounds prepared were screened against *Candida albicans*. In general no inhibitory effects were noted against this pathogenic yeast; however, 4, 4'-dichlorobenzhydrol completely inhibited growth at 140 micrograms per ml.

Summary. A number of miscellaneous compounds related to DDT have been screened for their abilities to inhibit the

growth of 5 select bacteria and yeasts. The alcohols were much more active than the corresponding ketones.

1. Backeberg, O. O., and Marais, J. L. C., *J. Chem. Soc.*, 1945, v1945, 803.
2. *Org. Synth. Col.*, 1941, v1, 90.
3. ———, 1943, v2, 520.
4. Buu-Hoi, Ng. Ph., Ng. Hoan, and Ng. D. Kuong, *Redueil des travaux chimiques des pays-bas*, 1950, v69, 1085.
5. Grummitt, Oliver, Buck, Allen H. and Becker, E. I., *J. Am. Chem. Soc.*, 1945, v67, 2265.
6. Grummitt, O., and Marsh, D., *ibid.*, 1948, v70, 1289.
7. Forrest, James, Stephanson, O., and Waters, W. A., *J. Chem. Soc.*, 1946, v1946, 333.
8. Mastagli, P., *Compt. Rendus.*, 1937, v204, 1656.
9. Minnis, W., *J. Am. Chem. Soc.*, 1929, v51, 2143.
10. Moore, J. E., Ph.D. Thesis. Oregon State College, 1953.
11. Montagne, P. J., *Recueil des travaux*, 1905, v24, 105.
12. ———, *Berichte*, 1915, v48, 1027.
13. Newton, H. P., and Groggins, P. H., *Ind. and Eng. Chem.*, 1935, v27, 1307.
14. Schnackenberg, H., and Scholl, R., *Berichte*, 1903, v36, 654.
15. Smith, M. I., *J. Pharm. and Exp. Ther.*, 1946, v68, 359.
16. Szybalski, W., *Science*, 1952, v116, 46.
17. ———, *Bact. Proc.*, 1952, v1952, 36.
18. Szybalski, W., and Bryson, V., *J. Bact.*, 1952, v64, 489.

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Failure of Y⁹⁰ to Escape from Skeletally-Fixed Sr⁹⁰.* (22001)

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In the evaluation of the toxicity of radioactive elements deposited in living animals it is essential to know how much energy is delivered to the irradiated tissues. When the radioactive element gives rise to daughter products of different atomic species possessing

physical and chemical properties different from the parent species, particular caution is necessary. This fact was recognized early in the work on radium toxicity when it was discovered that a large fraction of Rn²²² escaped from skeletally deposited Ra²²⁶ and was exhaled in the breath. It has also been found recently(1) that considerable ThX (Ra²²⁴)

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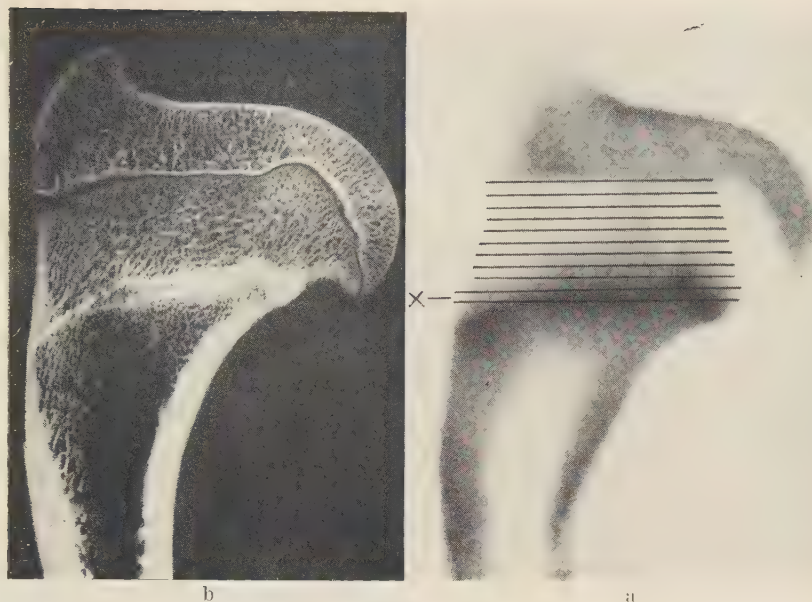


FIG. 1. a. Autoradiogram of slab of bone taken from proximal end of right humerus (magnification $1.5\times$). b. X-ray picture of same slab of bone at same magnification.

daughter escapes from parent RdTh (Th^{228}) deposits in bone. Sr^{90} is an important component of the radioactive fallout from atomic explosions. In considering its toxicity, particular interest is focused on the contribution to beta ray dosage which is made by the more energetic beta rays of its 2.7 day daughter, Y^{90} . A comparison of the energies of the Sr^{90} and Y^{90} beta rays shows that, under conditions of radioactive equilibrium, tissue would receive approximately four times as much energy from the more energetic beta rays of Y^{90} . Hence, we have studied the metabolic fate of the potentially more damaging Y^{90} formed from Sr^{90} deposits in the skeleton of a young beagle dog. The following are the possible metabolic pathways for the Y^{90} : (1) Excretion from the body; (2) Retention at sites of formation in the skeleton; (3) Escape from sites of formation and re-deposition in skeleton in different sites; (4) Some combination of the above.

Our experiments show that most or all of the Y^{90} daughter is retained at the sites of formation in the skeleton.

Methods. Our experiments were carried out on a single beagle dog which was sacrificed 4 months following the intravenous adminis-

tration of $150\text{ }\mu\text{c}$ per kg of Sr^{90} at equilibrium with its Y^{90} daughter. The injection solution consisted of Sr^{90} , Y^{90} in a citric acid, sodium citrate buffer of $\text{pH} = 3.5$ and 0.08 M total citrate. The dog was 5 months old at the time of injection, and therefore, its skeleton was growing during the presacrifice interval. The fate of the Y^{90} formed from Sr^{90} decay in the bone was studied in 3 ways. 1. The right humerus was cut longitudinally into slices 1-2 mm thick, embedded in Gelva resin,[†] and cemented on aluminum plates. After grinding the slabs flat with fine abrasive paper, contact autoradiograms were made on Kodak no-screen x-ray film. Fig. 1a is an autoradiogram (magnified $1.5\times$) of one of these slabs from the proximal end of the humerus; Fig. 1b is an x-ray picture taken at the same magnification. Another slab was cut along the parallel lines shown in Fig. 1a; adjacent lines are about 1 mm apart. The bone of section X was being deposited at the time of Sr^{90} injection and the bone of sections $X + 1$, $X + 2$, etc. was deposited at progressively later times. Following wet ashing, these samples, and also samples of bone from the shaft, were counted in a

[†] Shawinigan Products Corp., N. Y. City.

Geiger counter. Measurements were made both with and without an aluminum absorber sufficiently thick (0.7 mm) to absorb all Sr⁹⁰, but not all Y⁹⁰ beta rays. A series of such counts was made from 6 hours to 7 days after sacrifice. In all samples, the Y⁹⁰ beta ray activity was at equilibrium ($\pm 3\%$) with the parent Sr⁹⁰ at 6 hours after sacrifice.

Results. If Y⁹⁰ produced *in vivo* escaped from its site of formation into the general circulation, one would anticipate its concentration in the primary spongiosa beneath the epiphyseal line, in a manner similar to the deposition of parenterally administered Y⁹⁰(2). The amount of Sr⁹⁰ in the primary spongiosa is quite low since this was a growing dog and the bone of this area was deposited at a considerable time after injection of Sr⁹⁰. Because of this low Sr⁹⁰ concentration, an excess of Y⁹⁰ could have been readily detected. The fact that no such excess of Y⁹⁰ occurred in the primary spongiosa indicates that little, if any, of the Y⁹⁰ produced *in vivo* escaped from its sites of formation.

Fig. 1b also clearly demonstrates that metaphyseal trabecular bone containing a high concentration of radioactivity (section X) is markedly opaque to x-rays. This opacity is due to the presence of bone of abnormally high density in this region and is evidence of radiation damage. This point will be discussed further in a future publication. A similar effect can be seen in the radius of this dog (Fig. 2).

II. The left radius was divided into 8 segments as illustrated in the x-ray picture (Fig. 2). The pieces were quickly dissolved in nitric acid and aliquots were mounted for beta ray measurement. Aliquots of the samples were measured from 8 hours to one week after sacrifice by 2 methods. The Sr⁹⁰ and Y⁹⁰ beta rays were measured in a 2- π proportional counter; Y⁹⁰ only was measured using an end-window G-M tube with 0.7 mm aluminum between sample and detector. Similar measurements were made on the heads and shafts of the metacarpals. The data show no significant variation with time. The measurements of Sr⁹⁰ and Y⁹⁰ beta rays with time are rather insensitive, and the conclusion from these data is that the Y⁹⁰ activity at the time of sacrifice

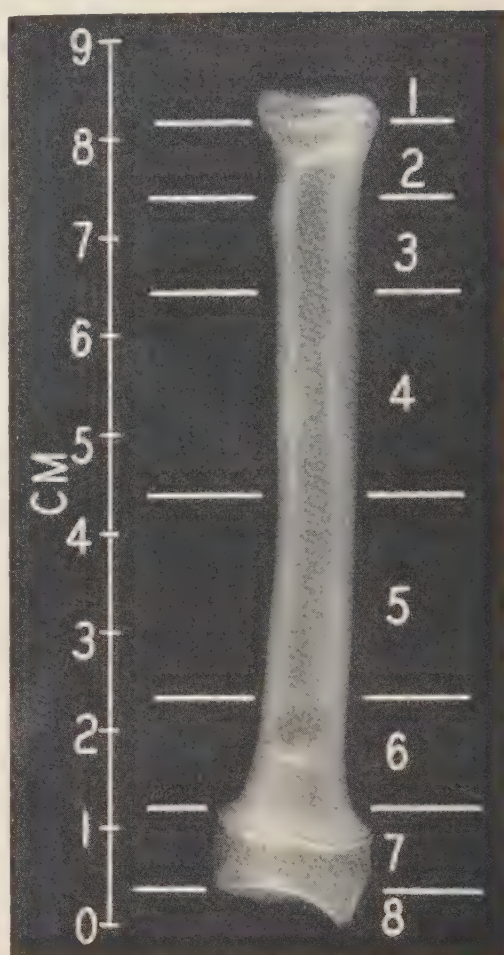


FIG. 2. X-ray picture of left radius.

equals the Sr⁹⁰ activity ($\pm 20\%$). The Y⁹⁰ beta ray measurements are more sensitive, and show that the Y⁹⁰ activity at the time of sacrifice equals the Sr⁹⁰ activity ($\pm 10\%$). Hence, Y⁹⁰ is close to equilibrium in these bone samples which differ both in Sr⁹⁰ concentration and morphologic composition.

Measurements were also made on the liver and plasma of this dog. The liver was homogenized, and an aliquot of the homogenate was wet ashed, as was a plasma sample. Aliquots of these samples were plated, and total beta ray activity was measured as described above.

In the plasma, Y⁹⁰ was not in equilibrium with its parent; the Y⁹⁰ activity was approximately half the Sr⁹⁰ activity. In the liver, Y⁹⁰ was in equilibrium, and the liver concentration

of Sr⁹⁰ was about equal to the plasma Sr⁹⁰ concentration. Since the skeleton contains approximately 5000 times as much Sr⁹⁰ as the liver, measurement of the liver for excess Y⁹⁰ is sufficiently sensitive to detect the escape of a small fraction of the Y⁹⁰ formed in the skeleton, even though the deposition in the liver of parenterally administered yttrium is low. If it is assumed that any Y⁹⁰ which leaves its site of formation in the skeleton behaves as parenterally administered tracer yttrium(3), then the lack of excess Y⁹⁰ in the liver shows that there is no appreciable escape from the skeleton of *in vivo* formed Y⁹⁰.

III. The x-rays produced by the Y⁹⁰ beta rays traversing the bony tissue of the right radius were measured as a function of time after sacrifice and of position along the axis of the bone. X-ray production by a continuous spectrum of beta rays of maximum energy E_{\max} striking a thick target is approximately proportional to E_{\max}^2 (4). Hence the yttrium x-ray production is approximately $(2.24/0.54)^2 = 17$ times as intense as the strontium x-ray production and is responsible for most of the counter response.

A $\frac{1}{4}$ inch separation between lead bricks was used to collimate the x-rays; the detector was a NaI(Tl) scintillation counter. Sufficient thickness of lucite was used to absorb all beta rays. A series of measurements at $\frac{1}{4}$ inch intervals along the length of the right radius was made. These measurements showed no growth or decay of x-ray production (to $\pm 5\%$) along the length of the bone. Since the x-rays detected were almost entirely

generated by Y⁹⁰ beta rays, these measurements show the presence of Y⁹⁰ rather than Sr⁹⁰. Hence, the Sr⁹⁰ in all segments of the right radius was close to equilibrium with the *in vivo* formed Y⁹⁰.

Summary. The data presented demonstrate that Y⁹⁰ produced *in vivo* from long term skeletal deposits of Sr⁹⁰ in young dogs does not escape from the local areas of bone in which it is produced. This is in agreement with previous experimental evidence(5). The implications of this finding are as follows: First, the combined energy of the Sr⁹⁰ and Y⁹⁰ beta rays irradiates the skeleton. Hence, dosimetric data determined *in vitro* can be directly applied to *in vivo* conditions. Second, the Sr⁹⁰ deposited in the growing skeleton is probably intracrystalline. This is in agreement with the x-ray diffraction findings of McDonald(6). Work is in progress to see whether this effect occurs in older dogs where there is less bone growth and less crystal formation.

1. Van Dilla, M. A., Stover, B. J., and Arnold, J. S., *Am. J. Roentgenol.*, in press.
2. Hamilton, J. G., *Rev. Mod. Phys.*, 1948, v20, 718.
3. Schubert, Jack, *et al.*, *J. Biol. Chem.*, 1950, v182, 635.
4. Introduction to the Atomic Nucleus, Robley D. Evans; MIT course notes to be published, Fall, 1955 by McGraw-Hill (N.Y.).
5. *Biological Hazards of Atomic Energy*, Ed. by A. Haddow, p. 152-153. Oxford Press, 1952.
6. McDonald, N., Ezmirlam, F., Spain, P., and McArthur, C., *J. Biol. Chem.*, 1951, v189, 387.

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Occurrence and Activation of an Elastase Precursor in Pancreas. (22002)

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Lansing and coworkers(1) have found that in teleost fish elastase occurs in pancreatic islet tissue but not acinar tissue. They cited, among reasons for studying this question, the findings that an herbivore (bovine) pancreas produces elastase and that the enzyme has not been detectable in human pancreatic juice. Kokas and coworkers(2) reported the presence of elastase in dog pancreatic juice, but occasionally it could not be found even when proteolytic activity could be measured.

Obviously, the establishment of an islet origin for elastase would indicate an endocrine function. Before an acinar origin and an occurrence in pancreatic juice could be unequivocally excluded, the possibility of an activatable zymogen was explored. The present study indicates that in dog pancreatic juice and in hog pancreas extracts the elastase does exist in the zymogen form, and a duodenal factor or trypsin is capable of converting this precursor to active elastase.

Methods and materials. Activations were carried out at 24°C for 20 minutes with no shaking. Each system contained 0.4 ml of dog pancreatic juice or hog pancreatic extract in a total of 1.4 ml. Other components and their concentrations per tube were: crystalline trypsin and crystalline α -chymotrypsin, 0.2 mg; crystalline soybean trypsin inhibitor, prepared according to Kunitz(3), 2 mg; amorphous pancreatic trypsin inhibitor, prepared according to Kazal, *et al.*(4), 2 mg; 0.4 ml of a 20% aqueous extract of the dried-defatted hog duodenum; and 0.02M Sorensen phosphate buffer, pH 7.4. The juice, which was kindly supplied by Dr. H. Necheles of Michael Reese Hospital, was collected by cannulation after the dog was treated with 100 units secretin, 3 mg mechoyl, and nembutal. The extract was a clarified 2% solution of a lyophilized 25% aqueous extract (pH 7.0) of fresh hog pancreas. The assay system was a modification of that of Baló and Banga(5). Elastin was prepared from beef aorta after removal of

collagen with boiling 1 N NaOH. The tubes contained 20 mg elastin, 0.5 ml of 0.5 M Sorensen phosphate buffer pH 7.4, 1.0 ml enzyme (activation mixture), and H₂O to 5.0 ml. Assays were performed for 30 minutes at 37°C with shaking. The tubes were chilled to 1°C, and their contents were filtered (S and S 595 paper). Total protein was determined in the filtrate by a Folin phenol method(6) in which crystalline bovine albumin was used as the standard.

Results. The activation studies are shown in Table I. Elastase is secreted in pancreatic juice in an inactive state capable of being converted to the active enzyme by trypsin or a duodenal factor but not by α -chymotrypsin. The duodenal effect is abolished by soybean and pancreatic trypsin inhibitors if the inhibitors are present from the beginning of the activation period. If present only during the assay they do not completely block the activated elastase. In control experiments, these inhibitors very effectively blocked the hydrolysis of denatured hemoglobin by trypsin; with an inhibitor to enzyme weight ratio of 1, the percent inhibitions by the soybean and pancreatic inhibitors were 87 and 80, respectively.

Discussion. The foregoing data point to an

TABLE I. Activation of Pro-elastase.

Activator	Elastase activity*	
	Dog pancreatic juice	Hog pancreatic extract
None	.7	.9
Trypsin	6.3	4.3
α -Chymotrypsin	.9	.0
Duodenal extract	5.8	3.7
Duodenal extr. + soybean trypsin inhibitor	.8	.4
<i>Idem</i> †	1.9	2.8
Duodenal extr. + pancreatic trypsin inhibitor	.6	.5
<i>Idem</i> †	1.3	2.6

* mg net solubilized protein.

† Inhibitors were added to activation tubes immediately before the aliquot was withdrawn for assay.

indirect role for duodenum—one of activating trypsinogen. Nascent trypsin appears to be the direct activating agent for pro-elastase. The absence of elastase activity in trypsin has been shown by Balo and Banga(5), and we have found it to be absent in both trypsin and α -chymotrypsin.

Several investigators have reported active elastase in extracts of desiccated pancreas without a duodenum component(5,7,8). These observations might be reconciled in the light of varying amounts of active protease in fresh mammalian pancreas tissue(9). Unless the starting tissue has an exceedingly low concentration of active trypsin or the investigator has taken special steps to avoid autoactivation, a given pancreas extract may be expected to contain sufficient trypsin to activate pro-elastase.

The data shown in Table I suggest that the duodenal factor is enterokinase. The activation mechanism strongly resembles that of chymotrypsinogen(10) and pro-carboxypeptidase(11) and may be represented as

(A) Trypsinogen $\xrightarrow{\text{enterokinase}}$ trypsin

(B) Pro-elastase $\xrightarrow{\text{trypsin}}$ elastase

Summary. Dog pancreatic juice and extracts of hog pancreas contain elastase in an inactive form. Trypsin or a duodenal factor is capable of activating pro-elastase. The activation is blocked by pancreatic and soybean trypsin inhibitors, an indication that the duodenal factor is enterokinase.

1. Lansing, A. I., Rosenthal, T. B., and Alex, M., *Proc. Soc. Exp. Biol. and Med.*, 1953, v84, 689.
2. Kokas, E., Foldes, I., and Banga, I., *Acta Physiol. Acad. Sci. Hung.*, 1951, v2, 333.
3. Kunitz, M., *J. Gen. Physiol.*, 1946, v29, 149.
4. Kazal, L. A., Spicer, D. S., and Brahinsky, R. A., *J. Am. Chem. Soc.*, 1948, v70, 3034.
5. Balo, J., and Banga, I., *Biochem. J.*, 1950, v46, 384.
6. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J., *J. Biol. Chem.*, 1951, v193, 265.
7. Banga, I., *Acta Physiol. Acad. Sci. Hung.*, 1952, v3, 317.
8. Pepler, W. J., and Brandt, F. A., *Brit. J. Exp. Path.*, 1954, v35, 41.
9. Robbins, K. C., Grant, N. H., and Schlueter, R. J., unpublished data.
10. Northrop, J. H., Kunitz, M., and Herriot, R. M., *Crystalline Enzymes*, Columbia Univ. Press, New York, 1948, 96.
11. Anson, M. L., *J. Gen. Physiol.*, 1937, v20, 777.

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Clostridium perfringens Iota Antitoxin Levels In Convalescent Sera from Hemorrhagic Fever Patients. (22003)

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The resemblance of the anatomic-morphologic changes in men who died of hemorrhagic fever (HF) to those in animals which succumbed to enterotoxemias caused by *Clostridium perfringens* led to an inquiry into the possible role of *Cl. perfringens* in the Korean variety of that human disease(1-4). The occurrence of *Cl. perfringens* in the human intestine is well known. Past studies concerned with the distribution of toxigenic types of *Cl. perfringens* have demonstrated that types A and F constitute all of the human intestinal isolates with a few exceptions(5-8). The

classification of the strains isolated from the colon of HF patients has not yet been undertaken due to the present limited supply of typing antitoxin on hand. It seemed, however, that the demonstration of serum antitoxins against known species of *Cl. perfringens* in convalescent HF patients would be another approach to this problem.

Materials and methods. *Sera.* Sera were obtained from 17 patients on the Hemorrhagic Fever Wards of an Army Hospital in Korea, during their convalescence from a disease answering the clinical criteria of HF. These

sera were drawn 35 to 51 days after onset of the disease and were compared with sera of 50 individuals from Japan and areas other than Korea who had never suffered such an illness. *Toxins.* Crude toxins were prepared from the following known type strains of *Cl. perfringens*: type A, No. 146 from Dr. L. S. McClung, Univ. of Indiana; type B, No. CN 667; type C, No. CN 882; type D, No. CN 1635; type E, No. CN 1241, and type F, No. CN 2048 from Dr. H. Warrack of the Wellcome Laboratory, Beckenham, England. The *Cl. perfringens* strains were grown in a horse muscle infusion containing 1% papain digest of horse muscle and 30% horse muscle particles, as recommended by Ross(9). The cultures were incubated for 18 hours at 35°C, centrifuged at high speed for 15 minutes to remove the large particles and sterilized by Seitz filtration. A later batch of type E toxin was sterilized by passage through a millipore filter with less loss of toxigenic material. All filtrates, except type A, were neutralized with alpha antitoxin to eliminate that toxin which is common to all recognized types of *Cl. perfringens*. The epsilon toxin of type D was activated with trypsin prior to use, as recommended by Batty and Glenney(10). This activation was not applied for the iota toxin of type E, as no enhancing value was noted after 7 hours incubation(9). The filtrates were tested against known antitoxins to confirm their type.* The L+ of each toxin was determined by the Ross method(9). *Procedure with HF sera.* Screening tests were performed by adding 0.6 ml sera which had been inactivated at 56°C for 30 minutes to 1.8 ml toxin containing 6 L+ mouse units, incubating the mixture for 30 minutes at room temperature and inoculating 0.5 ml of this mixture intravenously into each of 4 mice weighing 17-20 g. Neutralization was considered to occur if the mice survived for 72 hours. Quantitative determinations were performed as follows: The sera were diluted 1:2, 1:4, 1:6, 1:8 and 1:10 with physiological saline. One ml of each dilution was mixed with 1.2 ml toxin containing 6 L+ units and tested as in the screening

TABLE I. Individual Iota Antitoxin Levels of 67 Sera.

Units/cc serum	No. sera showing antitoxin	
	HF sera	Control
0 - .3	1	27
.4 - .9	0	14
1 - 2.9	1	9
3 - 4.9	0	0
5 - 6.9	8	0
7 - 8.9	4	0
9 - 11.9	2	0
12 - 21	1	0
Total	17	50

test. Control sera were tested in the same manner using undiluted and 1:2 diluted sera. All titrations were repeated on 3 different days to confirm the reproducibility of results.

Results. In the preliminary tests no neutralization of toxins produced by *Cl. perfringens* types A, B, C, D, or F was observed. Sera from 15 of the 17 HF convalescents contained sufficient antibody to neutralize the iota toxin produced by *Cl. perfringens* type E (Bosworth). Table I indicates the antitoxin titers of these 17 test sera as well as of the 50 control sera.

Sera tested against type E filtrates not neutralized with alpha antitoxin failed to protect the mice. Death was accompanied by hemoglobinuria which is common in lecithinase intoxication. This was not observed in mice killed with type E filtrate in which the alpha toxin was neutralized.

Discussion. The only major toxin produced by *Cl. perfringens* type E not formed by the other toxigenic strains is the iota toxin. Iota toxin is lethal, necrotizing, non-hemolytic and is activated by trypsin.

It is assumed that the neutralization observed was due to the presence of iota antitoxin as antitoxins of the other toxigenic types failed to protect mice when inoculated with type E filtrates used in these tests. The occurrence of iota neutralizing antibodies in the sera of convalescent HF patients in higher concentrations than in the 50 control sera is of interest. Further studies are being carried out to determine if an increase in iota antitoxin occurs during the course of HF. Cultural studies are also being conducted to determine the toxigenic types of *Cl. perfringens*

* *Cl. welchii* antitoxins supplied by Burroughs Wellcome and Company.

isolated from the stools of HF patients and from the soil of the endemic area in Korea.

Summary. Sera from 17 patients convalescing from hemorrhagic fever and 50 normal controls were tested for the presence of neutralizing antibodies of *Cl. perfringens* iota toxin. Hemorrhagic fever sera contained a significantly higher quantity of neutralizing antibodies than did the control sera.

We should like to express our thanks to Dr. McClung for the culture of *Cl. perfringens* type A, and to Dr. Warrack for the other toxigenic types, as well as for the type antitoxins made available by her from the Wellcome Research Laboratory.

1. Lukes, R. J., *Am. J. Med.*, 1954, v16, 639.

2. Bosworth, T. J., *J. Comp. Path.*, 1943, v53, 245.
3. Baldwin, E. M., Frederick, L. D., and Ray, J. D., *Am. J. Vet. Res.*, 1948, v9, 296.
4. Oakley, C. L., *Bull. Hyg.*, 1943, v18, 781.
5. Taylor, A. W., and Gordon, W. S., *J. Path. Bact.*, 1940, v50, 271.
6. Brothwick, G. R., *Brit. J. Exp. Med.*, 1937, v18, 475.
7. Taylor, A. W., and Gordon, W. S., *J. Path. Bact.*, 1940, v50, 271.
8. Hain, E., *Brit. Med. J.*, 1949, v1, 271.
9. Ross, H. E., Warren, M. E., and Barnes, J. M., *J. Gen. Microbiol.*, 1949, v3, 148.
10. Batty, I., and Glenny, A. T., *Brit. J. Exp. Path.*, 1947, v28, 110.

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Improved Procedure for Hypophysectomy of the Mouse.* (22004)

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As early as 1933, Selye, Collip and Thomson(1) reported the successful removal of the pituitary from female albino mice; no data on survival were included, although it was stated that mice hypophysectomized by the parapharyngeal approach tolerate the operation better than do rats. Four years later, Leblond and Nelson(2) published an extensive report on the histological changes observed in the adrenal, thyroid, and gonads of mice of the A and CBAN strains following hypophysectomy; a high mortality during the first post-operative week was reported, although the animals that survived this critical period subsisted thereafter apparently with no special therapy. Subsequently, Thomas(3) described a technic for which he used 2 minute hooks in place of the more customary dental drill to break through the cranium. The procedure for mouse hypophysectomy described in the present communication utilizes the basic tech-

nic of Smith(4), but introduces modifications which insure completeness of operation and make possible an operative survival approaching 100%.

Materials and methods. Materials. A cork-topped board of convenient size is outfitted with 2 rubber bands held taut by thumbtacks in order to secure the limbs. For immobilizing the head, a malleable wire is stuck firmly into the board and bent so that the incisors of the animal can be hooked securely beneath it. A 20-gauge one-inch injection needle bent at an angle of 120° serves admirably as a tracheal cannula. Cotton pledgets or miniature swabs are made by winding a small piece of cotton around the end of a pointed toothpick. These materials, together with a view of the operative setup, may be seen in Fig. 1. Other necessary equipment, forceps, dental drill, No. 5 dental burr, binocular magnifier mounted on a stand, and adjustable lighting, are similar to those required for the operation in the rat (5). Nembutal is used for anesthesia. 850 mg of sodium pentobarbital (USP) dissolved in 4 ml of propylene glycol, 2 ml of ethanol, and 14 ml of water serves as a stable stock

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FIG. 1. Operative setup for hypophysectomy of the mouse.

solution.[†] After the concentrated solution is diluted with distilled H₂O (1:4), injections are administered in a dose of 0.01 ml (0.085 mg of sodium pentobarbital) per g of body weight. *Operative technic.* Just before operation, the mouse is injected intraperitoneally first with the anesthetic agent and then with 0.25 mg of cortisone. The ventral neck region is shaved, a longitudinal midline incision, measuring 1 cm, is made, extending downward from the center of the lower jaw, and the sternohyoid muscle is separated so that the curved injection needle can be hooked gently into the trachea. Following cannulation, the base of the skull is reached by the usual parapharyngeal approach, as described for the rat (5). In the mouse, special care must be taken to distinguish between the synchondrosis itself and the anteriorly located transverse venous sinus. For the best exposure of the underlying pituitary, the hole drilled into the cranium should be so placed that one-third is above and two-thirds are below the occipito-sphenoidal syn-

chondrosis. The intact lobes are then gently aspirated from the hypophyseal fossa; strong suction must be avoided since it ruptures the protective membrane which separates the pituitary from the brain proper. Upon removal of the gland, a pledget is inserted into the hole and the skin is closed with a single suture. Bleeding is usually negligible, and the pledget is removed before the animal is resuscitated. *Postoperative care.* Immediately following operation, the animal is placed in an atmosphere of 95% O₂-5% CO₂ until it recovers. It is then injected intraperitoneally with 1 mg of terramycin dissolved in 1 ml of 5% glucose solution. A similar injection is given on the second postoperative day along with a subcutaneous injection of 0.25 mg of hydrocortisone. Most strains require no additional therapy. For the best assurance of survival, hypophysectomized mice should be kept in a room free from drafts, at a constant temperature of approximately 82° F. *Completeness of hypophysectomy.* Immature female mice of the BALB/c, A/He, C₅₇, C3H and Swiss albino strains were hypophysectomized at 35 days of age by the technic described above. One month later they were sacrificed and their adrenals, ovaries and uteri were examined by gross observation for evidence of atrophy. These organs were then removed, dissected free from surrounding tissue and weighed on a Roller-Smith torsion balance. The non-operated animals were treated in a similar manner.

The results are summarized in Table I. At the time of autopsy, the hypophyseal fossa was also examined with a binocular microscope for possible pituitary remnants. Serial histological sections of the region were not made, since nearly all the operated animals showed marked atrophy of the organs associated with hypophyseal function.

Discussion. The method outlined herein is a modification of the technic developed by Smith (4) for hypophysectomy of the rat. It has been used with success on 5 strains of mice whose body weight is not less than 12 g. In smaller animals, difficulties in cannulation, and bleeding from the cranium, have been encountered. Some workers prefer to dispense with tracheal cannulation and to

[†] The commercial solution of nembutal was found to be unsatisfactory.

TABLE I. Effects of Hypophysectomy on Female Mice Operated on at 35 Days.

Strain	Operative condition*	No. of animals	Body wt, g		Organ wt, mg			Width of tibial epiphyseal cartilage plate, μ
			Day of operation	At autopsy	Adrenal	Ovary	Uterus	
BALB/c	N†	10	15	19	5.4 \pm .2‡	5.1 \pm .04	45.7 \pm 9.3	103.5 \pm 2.2
	H	8	16	13	2.1 \pm .2	1.1 \pm .07	5.9 \pm .6	71.2 \pm 1.5
A/He	N	11	15	20	3.9 \pm .2	4.7 \pm .3	51.5 \pm 28.4	120.8 \pm 5.9
	H	10	16	13	1.5 \pm .1	1.1 \pm .06	5.3 \pm .7	60.2 \pm 1.1
C57	N	10	13	19	3.9 \pm .2	6.4 \pm .3	65.5 \pm 3.2	119.2 \pm 1.7
	H	8	15	14	1.5 \pm .1	1.1 \pm .1	4.9 \pm .5	48.5 \pm 2.3
C3H	N	7	18	23	4.0 \pm .2	10.1 \pm .2	85.1 \pm 9.1	119.8 \pm 2.8
	H	10	18	15	1.6 \pm .2	1.2 \pm .1	6.1 \pm .6	58.0 \pm 1.1
Swiss albino	N	4	19	23	8.7 \pm .01	7.0 \pm 1.6	50.1 \pm 8.7	123.2 \pm 2.6
	H	5	22	19	2.7 \pm .4	2.2 \pm .3	10.2 \pm 1.3	60.7 \pm 2.1

* All animals operated on at 35 days of age and sacrificed at 65 days.

† N = Nonoperated. H = Hypophysectomized.

‡ Mean \pm stand. error.

permit the animal to breathe intermittently; however, it has been our experience that the attendant manipulation precipitates rupture of the delicate blood vessels.

All mice of the different strains after hypophysectomy show similar retardation of general body growth, inhibition of skeletal growth, and a marked degree of atrophy of the adrenals, ovaries and uteri. However, strain differences do become apparent during the postoperative period. Mice of the C3H and A/He strains require resuscitation in an oxygen-CO₂ atmosphere if better than 50% survival is to be expected. In addition, the latter strain must be supplied with an adrenal steroid following the operation and at regular intervals thereafter. 50% of the hypophysectomized females of the A/He strain not injected with hydrocortisone died within the first 24 hours and 80% were dead within 48 hours, whereas none of the hypophysectomized mice treated with hydrocortisone succumbed; 0.25 mg of hydrocortisone given subcutaneously as a saline suspension every tenth day was found to be sufficient to maintain this

sensitive strain. C57BL, C3H, BALB/c and Swiss albino mice seem to survive indefinitely without further hormonal injections.

Summary. A practical procedure for mouse hypophysectomy by the parapharyngeal approach has been developed. Innovations include tracheal cannulation, resuscitation in an O₂ atmosphere, and hydrocortisone therapy. Mice of the A/He strain are particularly vulnerable to the effects of pituitary loss and will survive only if they are supplied with an adrenal steroid at regular intervals. On the other hand, mice of the C57BL, C3H, BALB c and Swiss albino strains do not require special postoperative handling.

1. Selye, H., Collip, J. B., and Thomson, D. L., *Proc. Soc. Exp. Biol. and Med.*, 1933, v31, 82.
2. Leblond, C. P., and Nelson, W. O., *Bull. d'histol. appl. à la Physiol. et à la Pathol.*, 1937, v14, 181.
3. Thomas, F., *Endocrinology*, 1938, v23, 99.
4. Smith, P. E., *Am. J. Anat.*, 1930, v45, 205.
5. Selye, H., *Textbook of Endocrinology*, 2nd ed., 1949, Acta Endocrinol., Inc., Montreal, p. 232.

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Fatty Liver Induced by Orotic Acid Feeding. (22005)

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In the course of a study of the effects of orotic acid, a normal component of milk, on the nutrition of young rats, it was observed that several animals developed fatty livers(1). The present study was undertaken to determine whether this effect can be consistently elicited. Since orotic acid is known to be the normal precursor of thymine and the methyl group of thymine is derived from the "C₁ pool"(2), it seemed possible that fatty livers induced by orotic acid derive from depletion of the available supply of methyl groups, analogous to the effects of nicotinamide(3) and glycocyamine(4). This possibility was tested by including various lipotropic agents in orotic acid containing diets.

Experimental. Male rats of a locally maintained strain(5), weighing 40-45 g, were placed on the various experimental diets, in groups of 10 rats each. The basal diet was as follows: casein 18%, salts(6) 5%, sucrose 75%, corn oil 2%. To each 2 kg batch of diet was added the following vitamin supplement: cod liver oil 10 g, thiamine hydrochloride 25 mg, inositol 500 mg, pyridoxine hydrochloride 25 mg, riboflavin 50 mg, calcium pantothenate 125 mg, niacin 500 mg, *p*-aminobenzoic acid 250 mg, methylnaphthoquinone 25 mg, biotin 2 mg. In addition, all diets contained 0.2%

of sulfadiazine to limit the activity of the intestinal flora. The diets of various groups, as shown in Table I, contained also the following special supplements: folic acid 5 mg/2 kilo, vit. B₁₂ 1 mg/2 kilo, methionine 0.8%, choline 0.3%, thymine 1%, uracil 1%. The animals were sacrificed after 28 days and liver fat was determined by a method described previously (7). The results are summarized in Table I.

Discussion. Although the mechanism remains obscure, it is apparent in Table I that the presence of orotic acid in the diet of the rat results in the accumulation of liver fat. Analysis of the lipid mixture from such livers revealed that the increase is almost entirely in the neutral fat component, much as in the fatty livers of choline-deficient animals. The fatty livers do not appear to relate to some general action of pyrimidines *per se*, since neither uracil nor thymine induced fat accumulation. The failure of folic acid, cobalamine, methionine and choline to prevent the liver fat accumulation induced by orotic acid feeding would appear to eliminate the possibility that orotic acid, by forcing thymine synthesis, creates so serious a drain on the "C₁" or methyl group supply that choline deficiency results. Indeed, the most heavily fatty livers observed in the entire series were

TABLE I. Effect of Orotic Acid and Lipotropic Factors on Liver Fat.

Orotic acid, %	Special supplement	Wt gain, g/day	Liver wt, g	Liver fat, % (wet basis)	
				Mean \pm S.E.	Range
0	0	2.9	7.07	5.8 \pm .8	4.3- 7.2
.2	0	2.8	6.02	9.2 \pm 1.7	5.2-13.8
.5	0	2.5	7.94	10.9 \pm 1.3	7.5-15.3
1.0	0	2.4	9.07	13.6 \pm 1.4	8.3-17.0
0	Folic, B ₁₂	3.0	6.50	5.9 \pm .8	3.9- 9.7
.2	<i>Idem</i>	3.1	7.57	4.9 \pm .6	3.6- 6.3
.5	"	3.1	9.70	9.7 \pm 1.9	5.5-17.3
1.0	"	2.8	9.50	10.1 \pm 2.3	5.8-14.8
1.0	Methionine	1.9	5.36	12.7 \pm 1.6	7.6-16.1
1.0	Choline	2.4	8.82	16.8 \pm 1.7	11.1-22.9
0	Thymine	2.6	6.73	5.5 \pm .5	4.2- 6.2
1.0	"	1.8	7.24	13.4 \pm 1.4	7.8-21.6
0	Uracil	2.8	7.23	4.8 \pm .7	3.3- 6.5

those exhibited by rats receiving both orotic acid and choline. No alternate explanation of the effect of orotic acid on the neutral fat content of the rat liver is apparent at this time.

Summary. 1. Inclusion of orotic acid in the diet of young rats results in fatty liver formation. 2. Uracil and thymine, known to be synthesized from orotic acid, are without effect on liver fat content. 3. This effect of orotic acid is not counteracted by lipotropic factors such as folic acid, cobalamine, methionine or choline.

1. Standerfer, S. B., unpublished data.
2. Buchanan, J. M., and Wilson, D. W., *Fed. Proc.*, 1953, v12, 646.
3. Handler, P., and Dann, W. J., *J. Biol. Chem.*, 1942, v146, 337.
4. Stetten, D., Jr., and Grail, G. F., *ibid.*, 1942, v144, 175.
5. Wolfe, J. M., Bryan, W. R., and Wright, A. W., *Am. J. Cancer*, 1938, v34, 352.
6. Hubbell, R. B., Mendel, L. B., and Wakeman, A. J., *J. Nutrition*, 1936, v31, 141.
7. Handler, P., *J. Biol. Chem.*, 1948, v173, 295.

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Production of Type Specific Antisera to Poliomyelitis Viruses in Rabbits.* (22006)

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The objective of this study was the production for experimental purposes of anti-poliomyelitis sera of high titer, with a high degree of type-specificity. The report of Hirst and Gotlieb(1) of the production of highly specific antisera to influenza viruses by intravenous injection of rabbits suggested that this approach might well serve our purpose. Levinson *et al.*(2) have reported the production of antisera capable of neutralizing poliomyelitis viruses, by intravenous or intramuscular injection of rabbits. They refer to earlier experience of other investigators in producing such neutralizing antibody in the serum of non-susceptible animals.†

The results reported here consist of an extension of the findings of Levinson's group, by the use of smaller doses of virus as antigen given by the intravenous route. In addition, a direct effect of the antisera alone on kidney cell culture has been observed. The

main objective was achieved by repeated intravenous injections of rabbits with small doses of each of two types of poliomyelitis viruses grown in tissue culture. The titer of antibody to the same type of virus as that used for antigen (homotypic) as well as to the other type of virus (heterotypic) have been determined by neutralization tests in tissue culture.

Materials and methods. Type 1-Brunhilde and Type 2-YSK poliomyelitis viruses (obtained from Dr. Joseph L. Melnick, Yale University) which had been grown in either monkey testicular or human uterine tissue culture were used as antigens to start immunization of rabbits and for a booster dose at 5 months. The 50% endpoints (negative logarithm) of the cytopathogenic action of the viruses in HeLa culture were approximately 5 and 4 respectively. For booster doses, at 6 months or more from the beginning of immunization, Type 1-Mahoney and Type 2-MEF1 viruses grown in monkey kidney culture (obtained from Connaught Laboratories, Toronto, Canada) were used. The 50% endpoints of viral action in monkey kidney culture were 6.4 and 5.3 respectively. Supernate from a lightly centrifuged culture served as

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† By personal communication, Dr. Hubert Malherbe in the laboratory of Dr. John F. Enders has also reported the production of neutralizing antisera to poliomyelitis viruses, by intramuscular injection of rabbits with virus incorporated in oily adjuvants.

TABLE I. Development of Type 1 Neutralizing Antibody in Serum of Rabbits after Repeated Intravenous Injections of Type 1 Poliomyelitis Virus.

Interval since beginning immun. (mo):	1	5¾	6¾	8¾	14½	15
" " last booster (wk):	—	3	2	1	—	1½
Total No. of doses:	6	7	8	9	9	10
Reciprocal of dilution of serum at 50% endpoint of viral effect						
Rabbit No. 10	20	30	400	1800	Dead	
11	20	5	110	600	14	180
12	20	Dead				
14	30	330	380	1600	140	680
Log ₁₀ ED ₅₀ Mahoney virus in test:	2.4	2.3	1.9	2.1	2.0	2.0

antigen for injection. In neutralization tests, the Mahoney and MEF1 viruses were used. Equal parts of virus and serum dilution to be tested were mixed and incubated for one hour at 35°C. 0.1 ml of the mixture was added to 0.5 ml nutritive medium in a tube containing one-week-old monkey kidney cells cultured directly on glass(3). (Screw-capped pyrex glass tubes which had been inoculated with approximately 50000 monkey kidney cells were received from Microbiological Associates, Bethesda, Md.) Occasionally, confirmatory tests were made in HeLa cell culture(5), usually 4 days after inoculation of pyrex glass test tubes with approximately 30000 cells. Final observation of viral action was made at 5 or 7 days, depending on the condition of the control cultures, without added virus. The amount of virus introduced in all but one neutralization test was within the range of 50 to 250 ED₅₀ (effective doses); i.e., 10^{1.7} to 10^{2.4} times the amount of virus at the 50% endpoint of cytopathogenic action of virus in simultaneous titration in normal rabbit serum. Dilutions of virus in titration and of antiserum for determining neutralization endpoints were made at 0.5 log increments. Titrations were done in 3 to 6 replicate tubes. 50% endpoints have been calculated according to Reed and Muench(4). Serum endpoints are reported as the dilution of antiserum that was mixed with an equal volume of virus. Some values given in the tables represent the geometric mean of endpoints found in 2 or 3 tests done on different days.

Immunization schedule. New Zealand white rabbits weighing 2-3 kg were injected intravenously. Two groups of 5 rabbits each

were given 6 doses of 2 ml tissue culture virus of Type 1 or Type 2 as 3 doses a week for 2 weeks. At 5 months from the beginning of immunization, a booster dose of 1 ml was given. At approximately 6, 7 and 8 months, 2 ml each, and at 15 months, 3 ml booster doses of virus grown in monkey kidney culture were given. The rabbits were bled by cardiac puncture before, and 1 month after the beginning of immunization. They were bled also from 1 to 3 weeks after each booster dose. No anaphylactic reactions were observed. The rabbits continued to gain weight during the course of immunization. Deaths which occurred were not related to time of injection.

Results of homotypic neutralization tests. In each neutralization test, a "normal" serum control was included. This consisted of several pools of serum from the same rabbits taken before immunization, mixed with the same amount of virus as in the test. When the normal serum pool was tested "undiluted" there was no evidence of neutralization. A more sensitive test for the presence of any neutralizing capacity of pre-immunization serum consisted of titration of virus mixed with undiluted serum, either compared simultaneously with virus titrated in Earle's solution or with the expected titer based on 21 titrations with Mahoney, and on 22 titrations with MEF1 virus. With Mahoney virus, 5 separate titrations in monkey kidney culture and with MEF1 virus, 4 titrations in monkey kidney (and 1 in HeLa culture) yielded the expected, or observed, equal titer. Thus there was no evidence of any neutralizing effect of the serum of these rabbits before immunization.

TABLE II. Development of Type 2 Neutralizing Antibody in Serum of Rabbits after Repeated Intravenous Injections of Type 2 Poliomyelitis Virus.

Interval since beginning immun. (mo):	1	5½	6½	8	14⅔	15
" " last booster. (wk):	-	3	2	1	-	1½
Total No. of doses (2 ml each):	0	6	7	8	9	10
Reciprocal of dilution of serum at 50% endpoint of viral effect						
Rabbit No. 2	30	280	1700	5600	Dead	
15	0	100	300	5000	2500	400 · 560
16	0	10	350	2800	2000	560 1000
17		30	Dead			
18		100	"			
Log ₁₀ ED ₅₀ MEF1 virus in test:	1.8	1.9	2.0	1.9	1.8	1.8

Table I shows the neutralizing capacity of anti-Type 1 serum vs. homotypic Mahoney virus. Antibody was demonstrable in all 4 rabbits at 1 month, the level rising with 3 booster doses up to 8¼ months after the beginning of immunization when endpoints of serum of the 3 surviving rabbits ranged from 600 to 1800. During a subsequent rest period of 6 months, endpoints fell although antibody was still demonstrable. A subsequent booster dose in the 2 remaining rabbits raised the endpoints to moderately high levels, although not quite up to the maxima observed at 8 months.

Table II gives the endpoints of neutralization of anti-Type 2 sera vs. homotypic MEF1 virus. All 5 rabbits showed an antibody response of from 10 to 100 by the end of the first month. With 2 or 3 subsequent booster doses, levels rose to give endpoints at 8 months of from 2000 to 5600. After a rest period of nearly 7 months, antibody fell to moderate levels in the 2 surviving rabbits and rose somewhat again after one more antigenic stimulus.

Results of heterotypic neutralization tests. Table III gives the results of cross neutralization tests. The sera of 3 rabbits before immunization with Type 2 poliomyelitis virus showed no effect on Type 1 virus. By 6½ months after beginning of immunization, after 2 booster doses, sera of 2 of 3 rabbits showed endpoints of 10 each, the third, 0. An additional booster by 8 months did not increase this cross reaction. (The homotypic endpoint ranged from 2000 to 5600 in these three samples of serum.) None of 3 anti-Type 1 antisera (see Table III) showed heterotypic antibody by nearly 6 months, after 6 doses of

virus plus a booster dose. After a second and third booster dose, at approximately 7 and 8 months, 1 (or possibly 2) of 3 sera showed a minimal amount of cross neutralization. The homotypic endpoints of these sera at 8¼ months ranged from 600 to 1800. Later samples of sera were not tested vs. heterotypic virus because of a complicating reaction which will be described in the next section.

Immuno-cytopathogenic effect of antiserum. Since the initial series of injections as well as the first booster dose in the rabbits consisted of virus grown in human uterus or in monkey testis, the possibility of a reaction of the antisera directly on the monkey kidney cells was not as great as if the virus had been grown in the same type of cells as that used in the neutralization test. Indeed, the antisera being studied for antiviral activity showed no direct effect on monkey kidney cells until the 7 and 8 months' samples, after the rabbits had received their first exposure to virus grown in monkey kidney culture as 1 and 2 additional booster doses. The serum of one (#10) of the 6 rabbits remaining showed cytopathogenic effect at a 1/6 final dilution of serum; i.e., 0.1 ml undiluted serum added to 0.5 ml culture fluid. After the third booster dose of monkey kidney grown virus at 15 months, sera of all 4 rabbits surviving produced an untoward effect on monkey kidney cells in culture. The reaction consisted of either lysis or agglutination of the cells, resulting in the extreme in their complete destruction or their falling off the wall of the test tube. The reaction is comparable to that observed when HeLa cells were exposed to antiserum prepared in rabbits by intravenous injection of HeLa cell constituents(6). The

TABLE III. Degree of Cross Type Neutralization of Anti-Poliomyelitis Sera Produced in Rabbits.

	Anti-Type 2 serum vs.				Anti-Type 1 serum vs.		
	Type 1 (Mahoney) virus				Type 2 (MEF1) virus		
Interval since beginning immun. (mo):	—	5½	6½	8	5¾	6¾	8¾
" " last booster (wk):	—	3	2	1	3	2	1
Total No. of doses:	0	7	8	9	7	8	9
	Rabbit No.				Rabbit No.		
	2	0	0	0	10	0	10
	15	0	0	10	11	0	2
	16	0	10	10	14	0	0
Log ₁₀ ED ₅₀ virus:		2.1	1.7	2.2		1.7	1.9
			2.4			2.8	

addition of neutral red at a final dilution of 1/100,000, observed to be compatible with healthy cells, helped to visualize the reaction. One antiserum (rabbit #14, 15 months' sample) was tested repeatedly, yielding an average 50% endpoint of 30 of anti-kidney cell reaction. The endpoint of homotypic virus neutralization by this serum was 680.

The possibility of a cytopathogenic action on cells in culture not due to virus must be taken into account when carrying out neutralization tests, especially with high concentrations of antiserum such as used in heterotypic tests. Unless the antiserum alone is shown to have no effect on the cells in culture, this anti-cellular reaction could be mistaken for lack of cross neutralization.

Summary. A simple and inexpensive method for producing antisera with a high degree of neutralizing capacity for poliomyelitis viruses has been described. By repeated intravenous injection of rabbits with small doses of poliomyelitis viruses grown in tissue cul-

ture, antisera with a high degree of homotypic with little or no heterotypic neutralizing capacity have been produced regularly. Such antisera develop also to a lesser degree the capacity to lyse or agglutinate the cells in culture, in the absence of virus. When testing for heterotypic neutralization, this reaction may mask cross neutralization.

1. Hirst, G. K., and Gotlieb, T., *J. Exp. Med.*, 1953, v98, 41.
2. Levinson, S. O., Milzer, A., Shaughnessy, H. J., Wolf, A. M., Janota, M., Vanderboom, K., Neal, J. L., and Morrissey, R. A., *Proc. Soc. Exp. Biol. and Med.*, 1954, v87, 111.
3. Morann, G. L., and Melnick, J. L., *ibid.*, 1953, v84, 558.
4. Reed, L. J., and Muench, H., *Amer. J. Hyg.*, 1938, v27, 493.
5. Scherer, W. F., Syverton, J. T., and Gey, G. O., *J. Exp. Med.*, 1953, v97, 695.
6. Mountain, I. M., *J. Immunol.*, in press.

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Effects of Reserpine and Chlorpromazine on Nest Building and Thermal Preference in Rats. (22007)

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Reserpine and chlorpromazine have somewhat similar effects on animal behavior. Both drugs depress general activity(1,2) and reduce responsiveness to environmental stimuli(3-6). Both drugs also tend to cause hypothermia (1,4,8). This study attempted to determine whether two forms of basically unlearned motor behavior related to low temperature could be influenced by moderate amounts of these drugs. Hypothermia resulting from drug treatment might be expected to cause preference for a warm environment and increased nest building in rats; but general sedation, if marked, would tend to depress both nest building and active search for the preferred thermal environment.

Methods. Twenty-four male albino rats of Wistar stock, 120-180 days old and 275 g average weight were used. Thermal preference was measured in an apparatus consisting of 2 interconnecting plywood units (5 x 5 x 32 in.) in which the temperatures could be independently regulated by means of strip heaters(7). The temperatures were set at 14-18°C (room temperature), and 32-36°C, alternated each day with respect to unit heated. Illumination was provided by an overhead light. Each animal was given a daily test in the apparatus lasting 4 minutes. Every 10 seconds during the last 3 minutes of the period a notation was made of the unit in which the animal appeared. Preference was measured in terms of the frequency with which the animal appeared in the warmer unit. Thus a score from 0-18 was possible. Animals were maintained in large nesting cages (24 x 18 x 12 in.) with food and water always available. Nesting material was provided in the form of 150 g of wood shavings and 50 g of paper stripping. Each evening previous nests were broken up and the nesting material scattered

throughout the cage. On the following morning the nests were rated independently by the experimenter and a second person, using the scale of Stone and Walker(9). Ratings range from 0 for no evidence of a nest to 5.0 for an excellent nest. Room temperature was reduced to 10-14°C during the night to promote nesting and raised to 25°C between nesting periods. The first part of the experiment was a pre-drug phase of 4 days. Groups of animals equated on a basis of nesting performance were then assigned to the experimental treatments indicated in Table I. During this phase, subcutaneous injections of the drugs were given one hour before start of the nesting period. Reserpine was given as .25% solution in a propylene glycol base, chlorpromazine as 2% solution in isotonic saline.

Results. The results for nesting and thermal preference are shown in Fig. 1. The data were treated by 2-way analyses of variance in which effects of each drug and their possible interactions could be segregated. Pre-drug data showed no statistically significant group differences but thermal preference behavior was selective in that all animals preferred the cooler unit.

As shown by Fig. 1, reserpine affected both thermal preference and nesting behavior much more strongly than chlorpromazine. On the 3rd and 4th days of reserpine treatment (groups R and ChR) nest building was effectively abolished ($p < .001$) and a reversed preference for the warmer environment was established ($.05 > p > .02$; significance of mean frequency above expected chance frequency of 9). Chlorpromazine (groups Ch and ChR) inhibited nesting following the first and second injections. After the second day the apparent slight depression of nesting was of doubtful statistical significance ($p = .05$). Chlorpromazine alone had no effect on thermal preference. Disturbed thermal preference was evident for several days after discontinuing re-

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TABLE I. Experimental Groups.

Group	N	Treatment	Dose
R	6	Reserpine	.5 mg/kg for 3 days; .25 mg/kg on 4th day
ChR	6*	Chlorpromazine	10 mg/kg for 4 days
		Reserpine	.5 mg/kg for 3 days; .25 mg/kg on 4th day
Ch	6	Chlorpromazine	10 mg/kg for 6 days; 15 mg/kg for 2 days
C	6	Isotonic saline	Comparable volumes to group Ch

* All data discarded for one animal that died after first inj.; data for a second animal that died on 5th day was included in the analyses.

serpine but nest building recovered within one day.

Weight changes during nesting periods were also measured. Declines in weight, presumably reflecting reduced food intake, accompanied the depressed nesting. Prompt recovery of weight followed discontinuance of reserpine injections.

ery of weight followed discontinuance of reserpine injections.

Discussion. Both reserpine and chlorpromazine affected nesting behavior and weight loss but, with the doses used, the effects of reserpine were clearly much greater. Reserpine

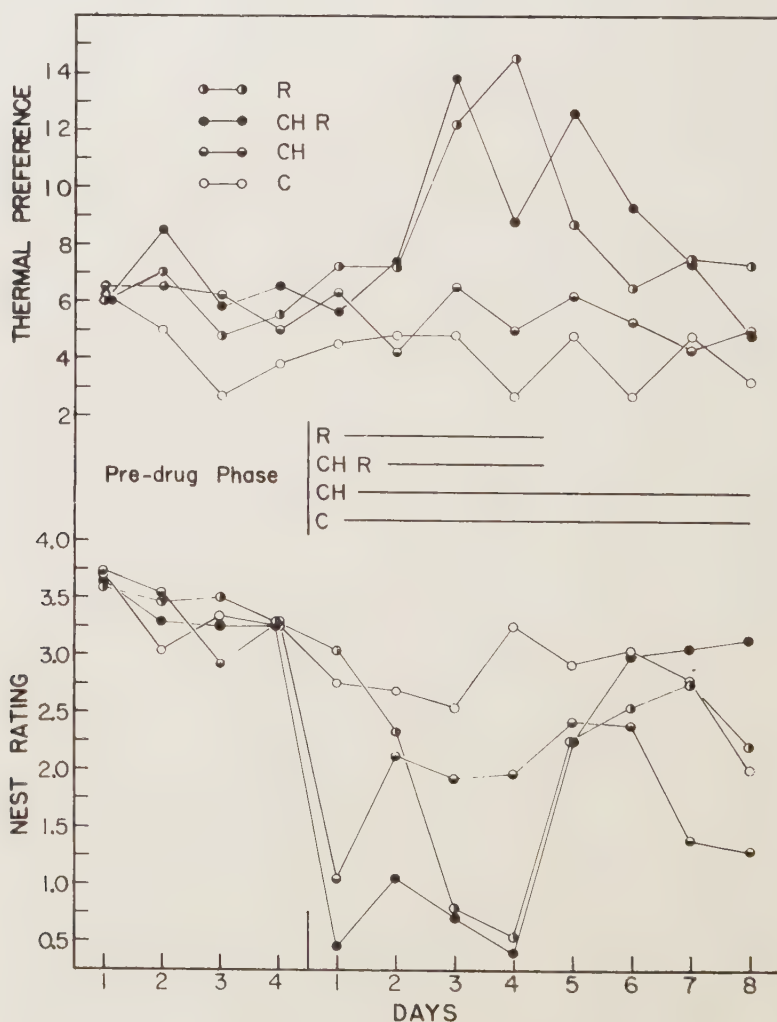


FIG. 1. Mean thermal preference scores and nest ratings.

alone affected thermal preference. Slight indications of synergic effects of the 2 drugs were seen but not statistically verified. The persistence of preference for a warmer environment for several days after discontinuing reserpine injections might be due to a persistent drug effect or to a persistence of a behavior learned during the drug phase.

It is of interest that reserpine greatly depressed one adaptive behavioral reaction (nest building) to the hypothermic state presumably resulting from the doses used but promoted another (choice of a warm environment). This difference may be related to the marked differences in the nature of the behavioral responses tested and the methods of testing. The thermal preference test involved arousal of the animal by handling, which could be expected to last throughout the 4-minute test period; nest building required long persistent activity under conditions of minimal disturbance. Reserpine-treated animals were easily aroused and quite active while awake but readily fell into a deep sleep when not disturbed. The experiment does not exclude, however, the possibility of a differential drug effect on mediational mechanisms of the two behaviors. The implications for behavioral research with reserpine in terms of distinguishing between sedative effects of the drug and other more specific actions are apparent.

Summary. Effects of reserpine, chlorpromazine and combined drugs on nest building and thermal preference of rats are described. In the doses used, reserpine alone had a marked and persistent effect on these functions. It depressed nest building while promoting choice of a warm environment. The differential behavioral effect is thought to be related to the great differences in persistence of activity required for the two tests and the ability of reserpine-treated animals to maintain alertness for brief periods after handling.

1. Tripod, J., Bein, H. J., and Meier, R., *Arch. Intern. Pharmacodynamie*, 1954, v96, 406.
2. Boyd, E. M., and Miller, J. K., *Fed. Proc.*, 1954, v13, 338.
3. Chusid, J. G., Kopeloff, L. M., and Kopeloff, N., *PROC. SOC. EXP. BIOL. AND MED.*, 1955, v88, 276.
4. Plummer, A. J., Earl, A. E., Schneider, J. A., and Barrett, W., *Ann. N. Y. Acad. Sci.*, 1954, v59, 8.
5. Schneider, J. A., *Bull. Med. Research*, 1954, v9, 10.
6. Schneider, J. A., and Earl, A. E., *Neurology*, 1954, v4, 657.
7. Mason, W. A., The effects of hypophysectomy and electroconvulsive shock on thermal preference and nesting in rats, Ph.D. Thesis, Stanford U., 1954.
8. Ripstein, C. B., Friedgood, C. E., and Solomon, N., *Surgery*, 1954, v35, 98.
9. Stone, C. P., and Walker, A. H., *J. Comp. Physiol. Psychol.*, 1949, v42, 429.

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Total Plasma Ascorbic Acid as Index of Stress in the Rat. (22008)

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One of the manifestations of physiologic stress is an increase in the production by the adenohypophysis of adrenocorticotrophin (ACTH)(1). A temporary rise in the concentration of total plasma ascorbic acid and plasma ascorbic acid following administration of ACTH in man has been reported(2).

As a preliminary to further experiments, it was deemed desirable to compare the sensitivity of this index of stress and possible ACTH titer in the blood of albino rats with

other such indices.

Methods. Animals in one group were anesthetized with sodium-ethyl-methyl-butyl-barbiturate (Nembutal) for 2 hours. Those in Group 2 were anesthetized with Nembutal for 2 hours and during this time non-activated electrodes were kept in contact with the left cervical sympathetic trunk just posterior to the superior cervical ganglion. The third group was treated like Group 2 except that the electrodes were activated so that the sym-

TABLE I. Average % Increase or Decrease following Treatment Indicated.

Component of blood	Nembutal only	Nembutal with non-activated electrodes	Nembutal with stimulation
Ascorbic acid	+14.15 (13)*	+17.97 (18)	+23.86 (15)
Eosinophils	- 10.39 (20)	- 25.50 (20)	- 42.89 (31)
Lymphocytes	- 31.27 (17)	- 37.55 (17)	- 43.84 (29)
Neutrophils	+55.26 (18)	+66.67 (17)	+56.22 (30)

* Figures in parentheses = No. of animals.

pathetic trunk was receiving .05 to .07 volt impulses of 20 milli-second duration from a square wave stimulator 20 times every second. Prior to attachment of the electrodes, a section of the vagus nerve in the region being stimulated was removed from animals in Group 2 and 3 to avoid death from cardiac or respiratory failure during the 2-hour period of treatment. Stimulated animals that did not exhibit sustained expansion of the pupil on the activated side were discarded. Blood counts and total plasma ascorbic acid determinations were made before and after treatment. Blood was usually taken from the tail. It was taken from the heart in only 4 cases. These 4 were giving excellent results but enough blood could not be obtained from the tail. The leucocyte count of blood taken from the heart differs from that of blood taken from the tail(3). The Roe and Kuether method of determining plasma ascorbic acid was used (4). Diet and temperature were maintained relatively constant. The experiments were started at the same time every day. Rats used were young adult males, all about the same age and weight, from the E. A. Doisy

TABLE II. Percent of Animals Treated Showing an Increase or Decrease in Blood Components.

Component of blood	Nembutal only		Nembutal with non-activated electrodes		Nembutal with stimulation	
	Incr.	Deer.	Incr.	Deer.	Incr.	Deer.
Ascorbic acid	100		100		100	
Eosinophils	40	60	10	90	10	90
Lymphocytes	12	88	12	88	7	93
Neutrophils	100		100		93	7

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Results. The 3 types of treatment outlined above produce temporary manifestations of stress, and thereby possibly of ACTH titer in the blood, demonstrable by each of the criteria used. Such manifestations are eosinopenia, lymphopenia, neutrocytosis and an increase in the total plasma ascorbic acid. The average percent changes in the eosinophil and lymphocyte counts and in the plasma ascorbic acid determinations, indicate that animals with non-activated electrodes attached were subjected to greater stress than were those receiving Nembutal only, and that activation of the electrodes caused the most severe stress (Table I). The average percent increase in neutrophils was not as great in the stimulated animals as it was in animals having non-activated electrodes attached (Table I). There-

TABLE III. Levels of Significance (P) of Differences between Methods of Treatment Indicated.

Component of blood	Nembutal only and Nembutal with non-activated electrodes	Nembutal with non-activated electrodes and Nembutal with stimulation	Nembutal only and Nembutal with stimulation
Ascorbic acid	.343	.105	.046
Eosinophils	.018	.015	<.001
Lymphocytes	.370	.393	.083
Neutrophils	.087	—	.809

fore, the neutrophil count is probably not a reliable index of ACTH titer in the blood. This conclusion is upheld by other workers (5).

From Table II it is seen that during 2 hours of treatment, all the animals in each of the 3 groups show an increase in plasma ascorbic acid, whereas a few from each group paradoxically show eosinophilia and lymphocytosis.

Thus it would appear that plasma ascorbic acid is the most reliable index of stress even though statistical analysis of the overall results (Table III) points to eosinopenia as the most sensitive indicator.

Summary. The effect of electrical stimulation of the cervical sympathetic trunk on the number of eosinophils, lymphocytes and neu-

trophils and on the total plasma ascorbic acid in the blood of the rat, indicates that the amount of total plasma ascorbic acid rises temporarily in these animals following this type of stress and that it is a reliable index of the severity of such stress and possibly of ACTH production following such stimulation.

1. Selye, H., *Canad. M. A. J.*, 1944, v50, 426.

2. Stewart, C. P., Horn, D. B., and Robson, J. S.,

Biochem. J., 1953, v53, 254.

3. Goldie, H., Jones, A. M., Ryan, H., and Simpson, M., *Science*, 1954, v119, 353.

4. Consolazio, C. F., Johnson, R. E., and Marek, E., *Metabolic Methods*, 1st ed., 1951, C. V. Mosby, Saint Louis, pp. 198-199.

5. Dougherty, T. F., and White, A., *Endocrinology*, 1944, v35, 1.

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Effect of Intrinsic Factor Concentrate on Vit. B₁₂ Absorption by Gastrectomized Rats. (22009)

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A recent attempt(1) to use normal rats to test the potency of an intrinsic factor preparation revealed only an inhibitory effect on the absorption of oral vit. B₁₂ by this test animal. Normal rats had been employed for this study which involved doses of 4.2 and 0.42 μ g of radioactive vitamin labeled with cobalt 60.

Normal rats do not develop a macrocytic anemia comparable to pernicious anemia, and may thus be considered *a priori* unsuitable for the evaluation of intrinsic factor potency. In humans, however, gastrectomized individuals (2,3) have been employed successfully to demonstrate intrinsic factor despite the absence of anemia or megaloblastic marrow. This suggested the possibility that gastrectomized rats would serve as appropriate test animals. Accordingly studies were undertaken to examine the effect of potent intrinsic factor concentrates upon the extent of absorption of radioactive vit. B₁₂ by gastrectomized rats. Furthermore, physiological doses of 200 m μ g and 50 m μ g of vitamin, and 2 different intrinsic factor concentrates, were investigated.

Methods. Adult rats of different strains weighing 200-300 g were used. Upon open ether anesthesia total gastrectomy was performed utilizing an end to end esophagoduodenostomy to restore intestinal continuity. Fine silk was used for sutures and ligatures

throughout. Total removal of the stomach was verified in each instance by histological preparation of a sagittal section through the removed organ. In general at least 10 days were allowed for recovery from the operation before transfer of the animals to the nutrition laboratory. During the first 10 days after operation, the animals were fed on a milk and bread diet and lost about 10% of pre-operative body weight. Subsequently, they were offered a basal soybean diet, and during test periods were given 10 g of this diet daily. **Materials.** Two commercially available intrinsic factor concentrates were employed. Preparation I was used only in preliminary study (Series A) with low specific activity vit. B₁₂-Co⁶⁰. Its clinical potency was not retested. Preparation II was of proven potency at 40 mg, as tested hematologically and by the Schilling method(4), and at 20 mg by the latter procedure. Its binding power was determined by the tracer-dialysis method(5) to be 250 m μ g/mg. The vit. B₁₂ labeled with Co⁶⁰ employed in the preliminary study had a specific activity of \approx 55 μ c/mg; and the activity of the second preparation, used in 3 additional definitive studies, was \approx 200 μ c/mg.

Method. Four series of experiments (A, B, C and D) were performed in such manner

TABLE I. Summary of Fecal Excretion Results—Gastrectomized Rats vs Controls.

Series	mμg B ₁₂	mg IF	Phase	Duration (days)	No. of animals		Group avg % of dose excreted	
					Gastrectomized	Control	Gastrectomized	Controls
A	200	0	I	5	1	2	107	71 ± 6
		10	II	4	1	2	109	95 ± 3
B	200	0	I	7	4	4	102.0 ± 5.8	84.9 ± 5.1
		3	II	2	4	4	96.4 ± 2.6	>82.9 ± 6.4
C	50	0	I	8	4	4	101.7 ± 4.1	62.4 ± 14.5
		15	II	4	4	4	100.5 ± 2.9	97.5 ± 2.8
D	50	0	I	6	6	6	99.4 ± 5.0	50.0 ± 13.6
		.2	II & III	6	5*	6	104.6 ± 3.0	71.0 ± 17.2
		1	III & II	6	4*	6	99.9 ± 4.7	98.6 ± 3.5

* One animal was found dead before conclusion of this phase.

that each rat served as its own control. After a 24-hour basal collection period, each animal received a 50-200 mμg of Co⁶⁰ vit. B₁₂ by stomach syringe, and feces collected daily until fecal radioactivity was negligible (Phase I). Then the same dose of tracer was administered simultaneously with a fixed amount of intrinsic factor concentrate, and collections continued as before (Phase II). In one series (D) a third phase involving administration of additional intrinsic factor was introduced. In this series, control and gastrectomized rats were each divided into 2 groups for Phase II, one group receiving 0.2 mg of concentrate, the second group receiving 1 mg. During Phase III, these dosages were reversed. In Series A, animals were fed milk to flush out their bowels at the conclusion of Phase I. Radioactivity measurements by gamma ray scintillation counting(1,5) were performed on feces homogenates in water suspension. Although stools were collected daily, several daily collections were combined for measurement in certain instances. Excretion was essentially complete in 3-4 days. No radiometric examination was made of rat organs.

Results of excretion measurements were calculated as "% of Dose." A large value signifies negligible absorption, while a low excretion indicates absorption. Basal periods are not reported since activities were zero. Basal corrections for Phases II and III were based upon the average activity (usually negligible) excreted for the 2 days immediately preceding these phases.

Group average excretion values (± average deviation from the mean) are compiled in

Table I for each of the 4 series. Also shown are the doses of vit. B₁₂ and intrinsic factor administered, the duration of each phase and the number of animals employed. Series D excretions listed for phases II and III actually represent averages for 0.2 and 1.0 mg intrinsic factor fed in both phases.

Discussion. In none of the 4 series did the gastrectomized rats absorb significant amounts of vit. B₁₂ within our experimental error, regardless of the presence of intrinsic factor; and in Series A, C and D, intrinsic factor reduced absorption by the control rats to essentially zero. This inhibitory effect of intrinsic factor is not shown in Series B control animals, in part because fecal collections during the second phase (plus intrinsic factor) was discontinued after the second day. Undoubtedly further excretion of radioactivity would have been noted had the experiment been continued. Furthermore, it is questionable whether complete inhibition could be attained in this experiment in view of the low ratio (3.75) of intrinsic factor binding capacity to B₁₂ dose.

Series C and D were performed with 50 mμg of radioactive vitamin since the percentage absorption would be greater than at the 200 mμg level, and because 50 mμg represents a physiological dose equivalent weight-wise to 10-12 μg per adult human. In these experiments it was established unequivocally that gastrectomized rats absorb only a negligible fraction of the oral vitamin, whether or not intrinsic factor is administered, and that intrinsic factor inhibits absorption of the vitamin by the controls.

In Series C, the quantity of intrinsic factor concentrate employed (15 mg) was disproportionately large, but was reduced in Series D to 1.0 mg and 0.2 mg, the latter amount being calculated just to bind completely the oral vit. B₁₂ dose (*i.e.* binding power 250 m μ g/mg or 50 m μ g/0.2 mg). Complete inhibition of absorption occurs even with 1 mg of concentrate; and at 0.2 mg inhibition is still evident though to a reduced extent. It appears that complete inhibition of vit. B₁₂ absorption occurs when the ratio (IF binding capacity)/(vit. B₁₂ dose) is approximately 5. Until such an excess capacity is established, inhibition increases gradually with increasing quantity of intrinsic factor.

Obviously, the rat, gastrectomized or normal, is not a suitable animal for testing intrinsic factor activity, which causes only a diminished absorption in normal rats and which has no effect on gastrectomized animals. It is possible that the rat can utilize only endogenous intrinsic factor and that intrinsic factor of foreign origin is ineffective and inhibitory. The behavior of the gastrectomized animals may also indicate that the stomach is the major seat of vit. B₁₂ absorption by the rat. These points are to be tested by experiments with rat stomach concentrate. If this concentrate proves to be effective, it will demonstrate species specificity in the action of intrinsic factor.

The livers of gastrectomized rats contain an abnormally low vit. B₁₂ content, as would be expected from their inability to absorb appreciable quantities of the vitamin orally.

On a weight basis, the rat appears capable of absorbing greater quantities than the human. Thus, in the absence of intrinsic fac-

tor, absorption from an oral dose of 50 m μ g amounts to 20-25 m μ g; and from 200 m μ g, absorption is 30 m μ g. The equivalent dose figures for a 60 kg human would be 10 μ g and 40 μ g, and the corresponding absorption would amount to 4-6 μ g. Actually the human absorbs(6) only 1.5 μ g from such oral levels.

Summary. 1. Gastrectomized rats and appropriate control animals were fed 50-200 m μ g cobalt-60 labeled vit. B₁₂ with and without intrinsic factor concentrates. Absorption was determined by scintillation counting of feces homogenates. 2. No absorption of vit. B₁₂ by the gastrectomized animals was noted. 3. Absorption by control animals was inhibited by intrinsic factor concentrate, the extent of inhibition increasing with the quantity of concentrate administered.

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1. Rosenblum, C., Woodbury, D. T., Gilfillan, E. W., and Emerson, G. A., *PROC. SOC. EXP. BIOL. AND MED.*, 1954, v87, 268.

2. Swenseid, M. E., Halsted, J. A., and Libby, R. L., *PROC. SOC. EXP. BIOL. AND MED.*, 1953, v83, 226.

3. Halsted, J. A., Gasster, M., and Drenick, E. J., *New England J. Med.*, 1954, v251, 161.

4. Schilling, R. F., *J. Lab. Clin. Med.*, 1953, v42, 860.

5. Rosenblum, C., Woodbury, D. T., and Reisner, E. H., Jr., *Proc. Second Radioisotope Conference*, Oxford, July 19-23, 1954, Butterworth Scientific Publications, London, 1954, v1, p287.

6. Glass, G. J. B., Boyd, L. J., and Stephanson, L., *PROC. SOC. EXP. BIOL. AND MED.*, 1954, v86, 522.

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Effect of Chlorpromazine upon Epileptic and Normal Monkeys.* (22010)

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The widespread use of chlorpromazine in treatment of disorders affecting the nervous system of man suggested the advisability of a study of its effects upon epileptic and normal monkeys.

Method. Chronic experimental epilepsy was produced in 13 *Macaca mulatta* (3-4 kg) by the application of alumina cream to a cerebral cortical area(1). Discs containing alumina cream were applied to a principal sensorimotor area in 10 monkeys; multiple injections of alumina cream were made into an occipital cortical area in 2 monkeys and into an anterior mesial temporal area in the remaining monkey. Cerebral arteries (anterior, middle cerebral or carotid) were ligated in some monkeys in an effort to modify the clinical epilepsy produced. Chlorpromazine in doses of 2 mg/kg or 10 mg/kg was injected intravenously within one minute and the animals observed subsequently for a period of 4 hours during which behavioral, clinical and electroencephalographic studies were made. A total of 40 test injections (spaced at approximately biweekly intervals) were given to the experimental group. Clinical epileptic seizures were activated by vigorous prodding for one minute or by the intramuscular injection of small doses (16 mg/kg or less) of pentamethylenetetrazole (Metrazol®). Five normal unoperated monkeys were treated with chlorpromazine on 14 occasions and served as controls. In this group the effect of vigorous prodding as well as of intramuscular Metrazol (32 mg/kg) was similarly tested(2).

Results. Profound changes in behavior following intravenous injection of chlorpromazine occurred in both epileptic and normal monkeys. Within 1-2 minutes the animals became quieter, less aggressive, lethargic, flaccid and hypokinetic. They were soon unable to maintain a sitting or standing posture, becoming

progressively more stuporous; eyelids remained drooped or closed, and increased salivation and tachypnea were frequently noted. On being touched, the monkeys would occasionally make feeble efforts to blink eyelids, open mouth or withdraw a stimulated limb. Jerking myoclonic type movements of the body, tail or extremities sometimes occurred. Epileptic monkeys occasionally exhibited a typical focal or generalized motor convulsion spontaneously during chlorpromazine treatment. Maximum behavioral changes lasted 30 to 60 minutes, subsiding gradually thereafter within the next 4 to 6 hours. Effects were generally more marked and sustained with the larger doses used.

Pronounced electroencephalographic changes occurred within 1-2 minutes after the intravenous administration of chlorpromazine. Diffusely distributed slow waves with frequency of 2 to 5 per second and maximum amplitude of 150 microvolts became dominant. Against this slow wave background, there was usually noted in epileptic monkeys a striking pattern of increased spike and sharp wave discharges (Fig. 1).

Among the epileptic monkeys treated with chlorpromazine, the intramuscular injection of 16 mg/kg or less of Metrazol was uniformly effective in provoking clinical epileptic seizures. In some animals of this group, while under the influence of chlorpromazine, a smaller dose of intramuscular Metrazol was sometimes effective in provoking a seizure than was necessary in the untreated state. Normal control monkeys exhibited no clinical epileptic seizures while under the acute influence of chlorpromazine despite the use of relatively large doses of intramuscular Metrazol (32 mg/kg).

Vigorous prodding of epileptic monkeys following chlorpromazine treatment usually resulted in the activation of clinical epileptic seizures. Normal monkeys similarly stimulated while under the effect of chlorpromazine

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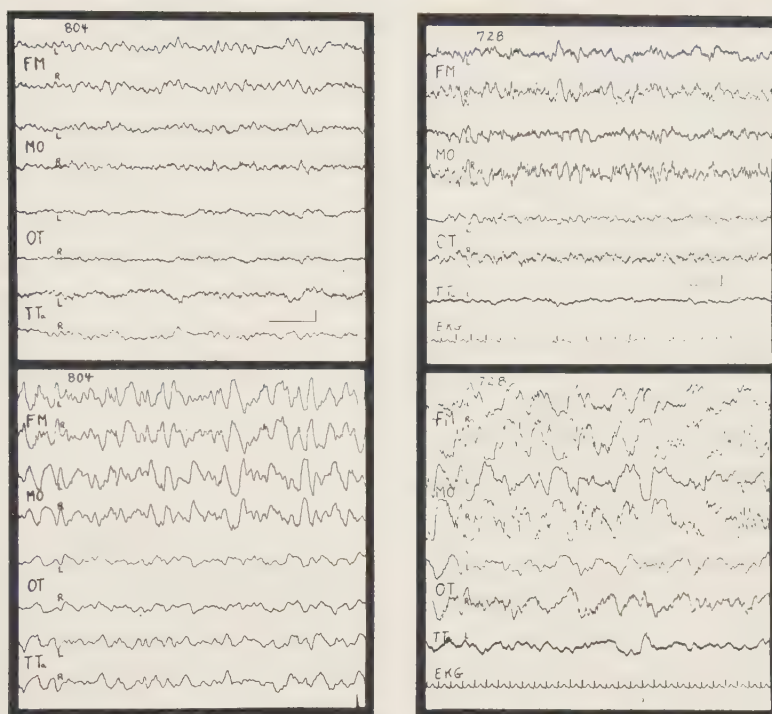


FIG. 1. Electroencephalogram before (top) and $\frac{1}{2}$ hr after (below) intravenous chlorpromazine treatment (2 mg/kg). Normal monkey (804) on left; epileptic monkey (728) on right. F = frontal; M = motor; O = occipital; T = temporal; Ta = anterior temporal; EKG = electrocardiogram. Calibration: 1 sec (horizontal); 50 microvolts (vertical).

never showed evidence of clinical seizures.

Discussion. In general the behavior of the epileptic and normal monkeys following the intravenous administration of large doses of chlorpromazine did not differ significantly, and resembled that described by Das *et al.* (3) for normal monkeys. Striking EEG changes noted in chlorpromazine-treated monkeys consisted of the prompt appearance of diffuse high amplitude slow frequencies. In the epileptic monkeys, there was, in addition, increased spike and sharp wave formations. This latter effect may be similar in type to that often encountered in man, where somnolence, or natural or drug induced light sleep increases the appearance of spike discharges (4). Chlorpromazine did not protect any epileptic monkey against the seizure precipitating effect of small doses of Metrazol. Some of these animals appeared to be even more susceptible since they required a smaller threshold dose to provoke clinical seizures. Normal monkeys, however, always remained refractory to intra-

muscular Metrazol even with the relatively high dose of 32 mg/kg (convulsant dose for normal untreated monkeys: 48 mg/kg or greater). Significant alteration in the clinical response of epileptic monkeys to activation of seizures by vigorous prodding was not observed in these experiments.

Conclusions. 1. The rapid intravenous administration of chlorpromazine (2-10 mg/kg) to epileptic and normal monkeys produced prompt profound behavioral changes characterized by hypokinesia, flaccidity, diminished responsiveness, reduced aggressiveness, lethargy and somnolence. 2. High amplitude slow wave activity (2-5/second) became dominant in the EEG up to several hours after intravenous injection of chlorpromazine; epileptic monkeys showed, in addition, increased high amplitude spike and sharp wave forms. 3. Epileptic monkeys under the acute influence of chlorpromazine retained their clinical convulsive responsiveness to intramuscular Metrazol (16 mg/kg or less). Clinical epileptic

response to vigorous prodding was not significantly changed. 4. Normal control monkeys under the acute influence of chlorpromazine did not exhibit clinical convulsions and remained refractory to treatment with intramuscular Metrazol (32 mg/kg) and to vigorous prodding stimulation.

Neurology, 1954, v4, 218.

2. Chusid, J. G., Kopeloff, L. M., and Kopeloff, N., *J. Appl. Physiol.*, 1953, v6, 139.

3. Das, N. N., Dasgupta, S. R., and Werner, G., *Bull. Calcutta School Tropical Med.*, 1954, v1, 5.

4. Gibbs, F. A., and Gibbs, E. L., *Atlas of Electroencephalography*, Addison-Wesley Press, Cambridge, Mass., 1952, v2, 19.

1. Kopeloff, L. M., Chusid, J. G., and Kopeloff, N.,

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In vitro and *in vivo* Neutralization of the Virus of Visceral Lymphomatosis. (22011)

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It has been reported(1) that the progeny of hens which had received a series of injections of the virus of visceral lymphomatosis were much more resistant to challenge with this virus than were the progeny produced by the same hens before the injections were made. The suggestion was made that the basis for this increased resistance was the transfer of antibodies from the dam to the egg and thence to the chick. This is known to occur in other diseases of chickens.

Tests reported here provide evidence that the tumor inciting properties of the virus of visceral lymphomatosis are neutralized by specific antiserum, that serum neutralizing antibodies in chickens are produced by the injection of materials containing the live virus, and that the increased resistance of the progeny is directly related to the increase in specific serum antibodies in dams.

Materials and methods. Antisera for *in vitro* and *in vivo* neutralization tests were obtained from hens that had received a series of injections of preparations containing the virus of visceral lymphomatosis. A description of these preparations and the sequence of injections have been given(1). Normal serum for both tests was collected 6 days before the hyperimmunizing injections were begun. Immune serum for the *in vitro* test was collected from one of the hens 4 days after the last of the first series of injections. Two months later a second series of injections was given intra-

muscularly. The material injected was live virus preparation similar to that used in the first series. Immune serum for the *in vivo* test was collected 6 weeks after the last of the second series of injections. There was an interval of about 6 months between the first injection and the final collection of immune serum. To provide a sufficient volume of serum for the *in vivo* test the serums from 9 hens were pooled. All serums were obtained from blood collected by heart puncture. The blood was transferred to large centrifuge tubes and refrigerated over night. After centrifugation the serum was transferred to glass vials and sealed. The tubes were stored at -30°C . The source of virus used in both tests was lymphomatous livers of birds in the sixteenth serial passage of strain RPL 12(2). The chicks were from an inbred line of S.C. White Leghorns maintained in isolation for the past 13 years. The incidence of lymphomatosis in isolation has been very low; however, when naturally or artificially exposed, the stock has manifested susceptibility to the disease. *In vitro* neutralization tests were made with constant volumes and dilutions of serums mixed with 4 serial 100-fold dilutions of the virus preparation. Dilutions were made with Simms' salt solution containing 1.5% bovine albumin. Each inoculating dose of 0.2 ml contained filtrate obtained from log -3 , -5 , -7 , and -9 g of lymphomatous liver. All serums were heated to 56°C for 30 minutes,

TABLE I. *In Vitro* Neutralization of Virus of Visceral Lymphomatosis by Specific Antiserum.

Serum	Log dose of virus	No. inoculated		% visceral lymphomatosis—				Latent period (days)
		Total	Corrected	112	140	168	201 days	
Normal	-3	42	40.7	61.5	66.4	69.8	71.3	81.0
	-5	40	35	17.1	31.4	37.2	60.0	138.2
	-7	37	36.1	8.3	24.9	33.3	36.0	129.5
	-9	41	39.4	0	0	2.5	12.7	191.8
Immune	-3	41	35.2	11.3	22.7	56.9	76.8	148.0
	-5	42	40.1	2.5	2.5	5.0	25.0	172.5
	-7	41	39	0	2.6	5.1	5.1	149.0
	-9	40	39	0	0	0	0	—
"	0	38	38	0	0	0	0	—
Diluent	0	38	38	0	0	0	0	—

24 hours before use. One ml of serum was added to each 9 ml of virus preparation. The virus preparation and the virus-serum mixtures were maintained at melting ice temperatures at all times. The interval between mixing and inoculation of the various mixtures was 2-3 hours. The inoculations were made with a 1 ml tuberculin syringe into day-old chicks by the intraperitoneal route. For *in vivo* neutralization tests, chicks were injected with pooled serum—on the first day with 0.5 ml intraperitoneally, on the second day with 1 ml subcutaneously, and on the third day with 0.5 ml subcutaneously. On the third day the chicks received by the intraperitoneal route 0.2 ml of the virus preparation of a dilution equivalent to log -3, -5, or -7 g of tumor. All chickens were maintained for a period of 201 days under experimental conditions similar to those previously described(3,4).

Results. Data obtained from the *in vitro* neutralization test are summarized in Table I. The percentage of mortality from visceral lymphomatosis was based on the number per lot as corrected(3) for deaths due to other causes. The incidence of tumors at progressive ages is given in the table, since it has been shown(4) that differences in response to different dosages tends to decrease with an increase in the holding period. With one exception, the percentage of visceral lymphomatosis for each age interval and for different virus dosage lots was lower in chickens that had received the inoculum containing immune serum than in those that had received mixtures containing the normal serum. These quantal response data clearly indicate that at the dose of log -9 practically all, if not all, of

the virus was neutralized and at the doses of log -7 and -5 at least 99% of the virus was neutralized. There was no essential difference in the incidence of tumors between the immune and normal serum at the log -3 dosage level and longest holding period, but at this highest virus dosage, partial neutralization is indicated by a marked delay in mortality. If the latent period is used as the basis for virus activity, it can be estimated that the addition of immune serum to the virus at a log dose of -3 had an effect equivalent to a dilution of the virus of at least 1:1000, or that neutralization of 99.9% of the virus occurred. This estimate was based on dose-latent period curves of 4 studies reported(4) and a fifth recently completed.

Data on the influence of injecting normal and immune serum into susceptible chicks prior to their being inoculated with various doses of virus are summarized in Table II. The mortality from visceral lymphomatosis to 201 days of age among chicks that received the normal serum injections was about the same as among chicks that did not receive any serum. The response in all of the three different dosage lots was less than expected on the basis of other inoculation results(4) with the same virus preparation. However, the responses were much greater than obtained with any of the 3 dosage lots in chicks injected with immune serum. Thus, none of the chickens that had received a total of 2 ml of immune serum during the first 3 days of life, and subsequently were inoculated with virus at a dosage of log -5 and log -7, and only 3 of 24 chickens similarly treated and challenged with the maximum dose of log -3, developed vis-

TABLE II. *In Vivo* Neutralization of the Virus of Visceral Lymphomatosis by Specific Antiserum.

Serum	Log dose of virus	No./lot		% visceral lymphomatosis at 201 days	Latent period (days)
		Total	Corrected		
0	-3	22	20.9	47.8	92.7
	-5	24	22.1	13.6	144.8
	-7	23	22.2	13.6	171.3
Normal	-3	23	20.7	63.8	83.1
	-5	23	21.4	14.0	157.1
	-7	24	23.4	14.0	144.3
Immune	-3	24	23.5	12.7	188.0
	-5	24	23.3	0	—
	-7	23	22.9	0	—
Non-inoc. controls		39	38.2	0	—

ceral lymphomatosis. Furthermore, these 3 died late in the experimental period, resulting in a mean latent period greater than that of any other lot.

Estimates of neutralization based on the latent period data of the *in vivo* experiment, are not nearly as reliable as those based on the *in vitro* experiments, because of the small number of birds that died; however, the differences are of such magnitude that they add considerable weight to the quantal response data.

Results from the 2 experiments described demonstrated the presence of an antibody which neutralizes the oncogenic activity of the virus of visceral lymphomatosis. This antibody was found to be present in serum of chickens after they had received a series of injections of filtered extract of lymphomatous liver containing the active virus, and was essentially absent in serum of the same chicks collected just prior to the injection of the virus. These chickens were from a flock that had been reared in isolation for many years and were relatively free from infection with any known disease agent.

There have been many reports [see review of Olson(5), and the reports of Kabat and Furth(6), Furth and Kabat(7)] of indications of immunity and of serum antibodies in experimental work with the leukemic forms of avian leukosis. Recently, Eckert *et al.*(8) working with the virus of myeloblastosis were regularly able to provoke the formation of specific neutralizing and precipitating antibodies in normal growing chickens not previously exposed. This was done by repeatedly

administering virus concentrates inactivated by formalin and then injecting highly infectious plasma. Lee(9) reported the development in ducks and turkeys of neutralizing antibodies effective against myeloblastosis and neurolymphomatosis. There is indirect evidence to indicate that the various forms of avian leukosis are caused by different etiologic agents(10-12).

Summary. Evidence is presented for the production of antibodies which neutralize the oncogenic activity of the virus of visceral lymphomatosis, by hens injected with virulent virus. Serum from such hens injected into chicks has produced a passive immunity.

1. Burmester, B. R., *Proc. Soc. Exp. Biol. and Med.*, 1955, v88, 153.
2. Burmester, B. R., and Cottral, G. E., *Cancer Res.*, 1947, v7, 669.
3. Burmester, B. R., and Gentry, R. F., 91st Ann. Meet. Am. Vet. Med. Assn. Proc., 1954, pp311-316.
4. Burmester, B. R., *Poultry Sci.*, in press.
5. Olson, Carl Jr., *Mass. Agr. Exp. Sta. Bull.* 370.
6. Kabat, E. A., and Furth, J., *J. Exp. Med.*, 1941, v74, 257.
7. Furth, J., and Kabat, E. A., *ibid.*, 1941, v74, 247.
8. Eckert, E. A., Sharp, D. G., Beard, Dorothy, Green, I., and Beard, J. W., *Proc. Soc. Exp. Biol. and Med.*, 1955, v88, 181.
9. Lee, C. D., *Am. J. Vet. Res.*, 1942, v3, 336.
10. Beard, J. W., Sharp, D. G., and Eckert, E. A., in press.
11. Burmester, B. R., Gentry, R. F., and Waters, N. F., *Poultry Sci.*, 1955, v34, 609.
12. Waters, N. F., *ibid.*, 1954, v33, 365.

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Antigens from Yeast Phase of *Histoplasma capsulatum*.—II. Immunologic Properties of Protoplasm vs. Cell Walls. (22012)

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Antigens from the yeast phase of *Histoplasma capsulatum* have been shown to enter into several immunologic or serologic reactions. In complement-fixation tests for detection of antibodies in experimental and naturally occurring histoplasmosis, antigens have included whole cells(1,2) and supernatant fluid from ground cells(3). In precipitation tests, a filtrate from the growth of cells of the yeast phase in broth has been used extensively(4). These complex antigens are of definite value as aids in diagnosis, although some cross-reactions occur with sera from other mycoses, primarily blastomycosis and coccidioidomycosis(5-7). Recently, attempts have been made to improve the specificity of the yeast-phase antigens(8,9). An endotoxin was demonstrated in formalin-killed whole cells of the yeast phase by intraperitoneal injection into 21-day-old white Swiss male mice(10). Inoculation of similarly prepared cells of the yeast phase in smaller quantities induced the development of resistance against subsequent lethal intracerebral or intraperitoneal challenges(11,12). Reactions of tuberculin or delayed type in sensitized individuals after injection of yeast-phase antigens are well known(13). Recently, it was also reported that hypersensitive mice could be killed in less than 48 hours by injection of dead whole cells in 5% mucin(14). Hemolysins that were most active against hen and guinea pig erythrocytes were demonstrated in soluble extracts from the yeast phase(15). Until recently, it had been believed that each cell of the yeast phase was enveloped in a capsule(16). Studies with the electron microscope, however, showed that these cells did not have a capsule(17). Since whole cells had been proven to be useful and active as antigens, the question arose as to where in the cell each antigen was located.

In this paper, methods are described for the

separation of cell walls from the protoplasm and data are presented illustrating some of the antigenic properties of these two fractions.

Materials and methods. White Swiss male mice, 21 days old and raised at the Rocky Mountain Laboratory, were employed. One isolate of *H. capsulatum* was used throughout, namely #6515, which originally had been isolated from a dog. It was maintained at 37°C on an agar medium consisting of 51 g/1 Difco cystine heart agar and 21 g/1 Difco bacto-hemoglobin. The yeastlike cells for preparation of antigen and for injection into animals were grown for 3 days under constant rotation at 37°C in a liquid medium containing salts, dextrose, and amino acids(12). The immunologic and serologic tests were similar to those previously described(1,18). *Preparation of Antigens.* Antigens were prepared by one of two methods, both of which included vibration with glass beads (#1241 glass beads, obtained from Cataphote Corp., Toledo, O.) in a container of a Mickle tissue disintegrator (manufactured by H. Mickle Co., Hampton, England). The time necessary for exposure of cells of the yeast phase to vibration was determined with the aid of an electron microscope. Varying concentrations of cells were exposed for varying periods of time in the tissue disintegrator. That time and concentration were used for separation of protoplasm from the cell wall wherein the cells were shattered sufficiently for the protoplasmic contents to escape but not enough for the cell walls themselves to be fragmented. For example, many of the ether-killed cells were suspended in 8 ml quantities (10^{10} cells/ml) with 8 g glass beads and vibrated for 10 minutes at 4°C.

Method 1. A cell suspension of the yeast phase was shaken with approximately 3 volumes of ethyl ether in large separatory funnels. At first, the cells sank to the bottom of

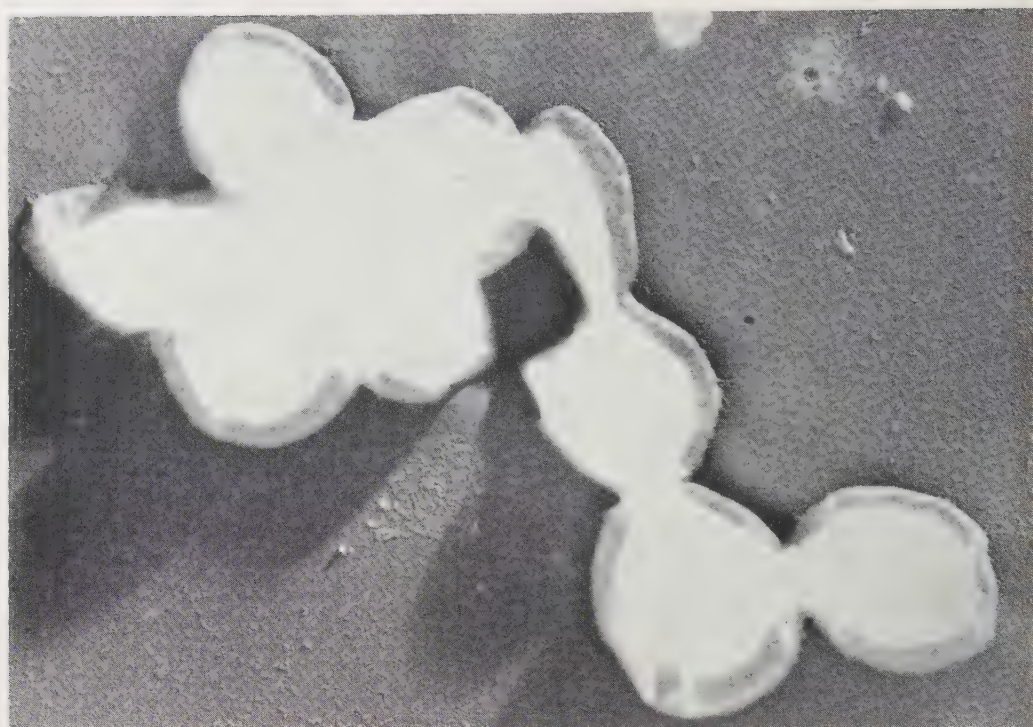


FIG. 1. Water-washed cells of yeast phase of *H. capsulatum*. Magnification 20000 \times .

the aqueous portion of this mixture. However, after several shakings over a period of approximately 24 hours, the cells rose to the water-ether interface. Cells were then harvested, washed by centrifugation in distilled water and reshaken with 3 volumes of ether. When cells of the yeast phase once again rose to the interface, they were washed and treated again in ether. After a third washing in distilled water, the cells were exposed to vibration in the Mickle tissue disintegrator. Subsequent washings by centrifugation in distilled water at 2500 g at 4°C produced two fractions: (a) the cell walls and (b) the supernatant protoplasm. The second fraction was filtered through a fine sintered glass filter to remove possible cell-wall fragments. Both fractions were subsequently lyophilized. *Method 2.* Cell suspensions from individual flasks were pooled and the cells washed by centrifugation in distilled water. Cells were then suspended in 1:5000 merthiolate for 5 days at 37°C, then the cells showed no signs of growth when cultured on Sabouraud's agar slants. The cells were washed in distilled

water and exposed to vibration in the Mickle disintegrator. After washing of resulting cell residue and filtration of protoplasm, the two fractions were lyophilized. Similar results were obtained in various immunologic tests described below with fractions prepared by these two methods.

Results. Morphology. The morphology of cells of the yeast phase of *H. capsulatum* was examined under the electron microscope after each step in the fractionation procedure. This examination was of great importance in procedures associated with disruption of cells by mechanical agitation and the separation of disrupted products by centrifugation. Representative pictures are included to illustrate changes in morphology (Fig. 1-4).

Fig. 1 shows the original morphology of the yeastlike cell as seen in preparations of cells suspended in water. A purified cell-wall preparation obtained from ether-killed cells is shown in Fig. 2; the corresponding filtered protoplasm, in Fig. 3. Fig. 4 demonstrates the unfiltered supernatant material after merthiolate-killed cells had been cracked in

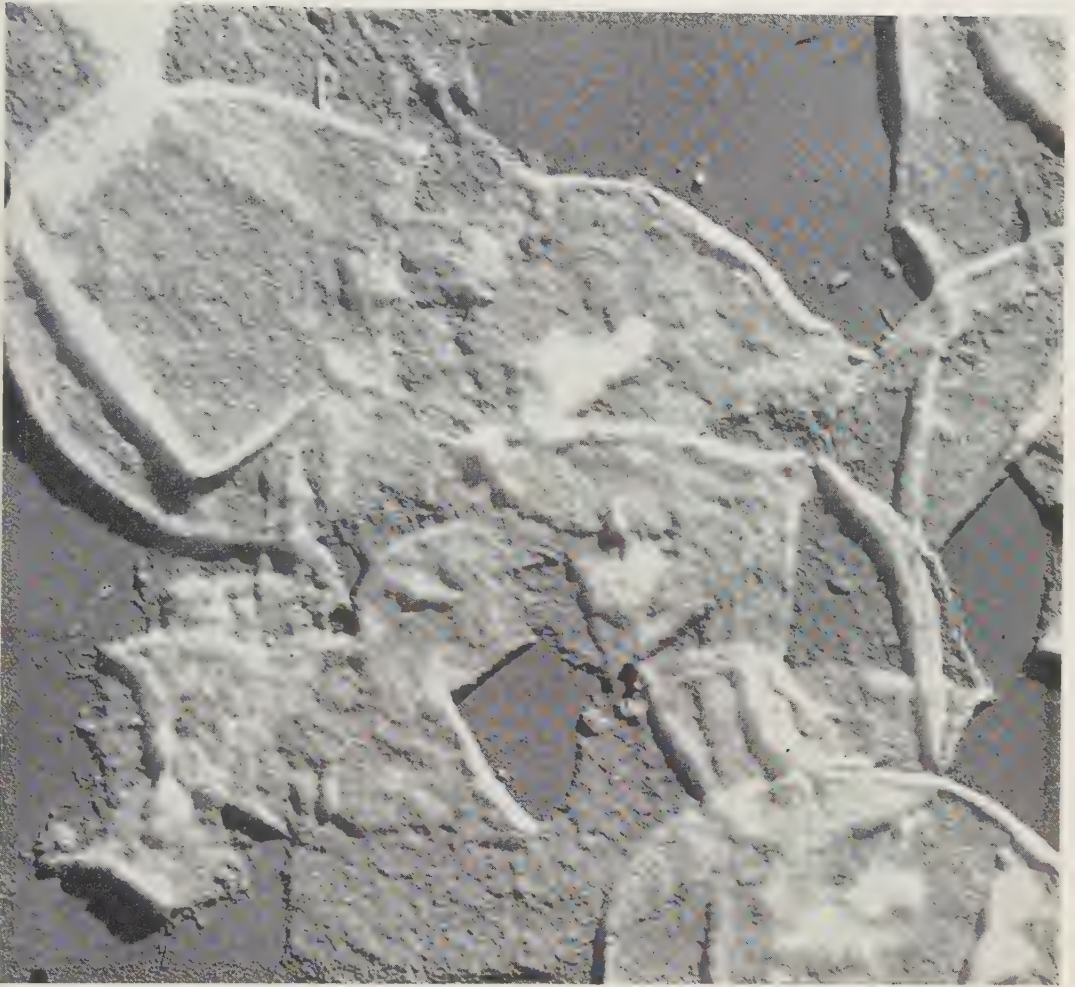


FIG. 2. Cell walls of ether-killed yeastlike cells of *H. capsulatum*. Magnification 44000 \times .

the Mickle disintegrator and centrifuged at 2500 g at 4°C for 30 minutes. Some cell-wall material was invariably present with the soluble protoplasm. The picture demonstrates the striking contrast in morphology between cell wall and protoplasm, and as a result the ease with which the two fractions may be distinguished under the electron microscope.

Examination of the protoplasm in Fig. 3 and 4 indicates that protoplasm of merthiolate-killed cells is released in a very fine, uniform, dispersed state, which condition permits easy separation of cell walls by centrifugation and filtration. In contrast, the protoplasm of ether-killed cells tends to contain more insoluble nondispersible granules, the larger of

which are separated from cell walls by centrifugation only with difficulty.

Complement Fixation. The complement-fixing activities of cell walls and protoplasm from ether-killed or merthiolate-killed cells were compared with the activity of heat-killed, lyophilized whole cells in the presence of human sera from proved or suspected cases of histoplasmosis and in the presence of sera from experimentally infected guinea pigs and rabbits. The cell walls had much more complement-fixing activity against human sera than an equal weight of whole cells. The filtered protoplasm, on the other hand, had much less activity than an equal weight of whole cells. The optimum level for whole

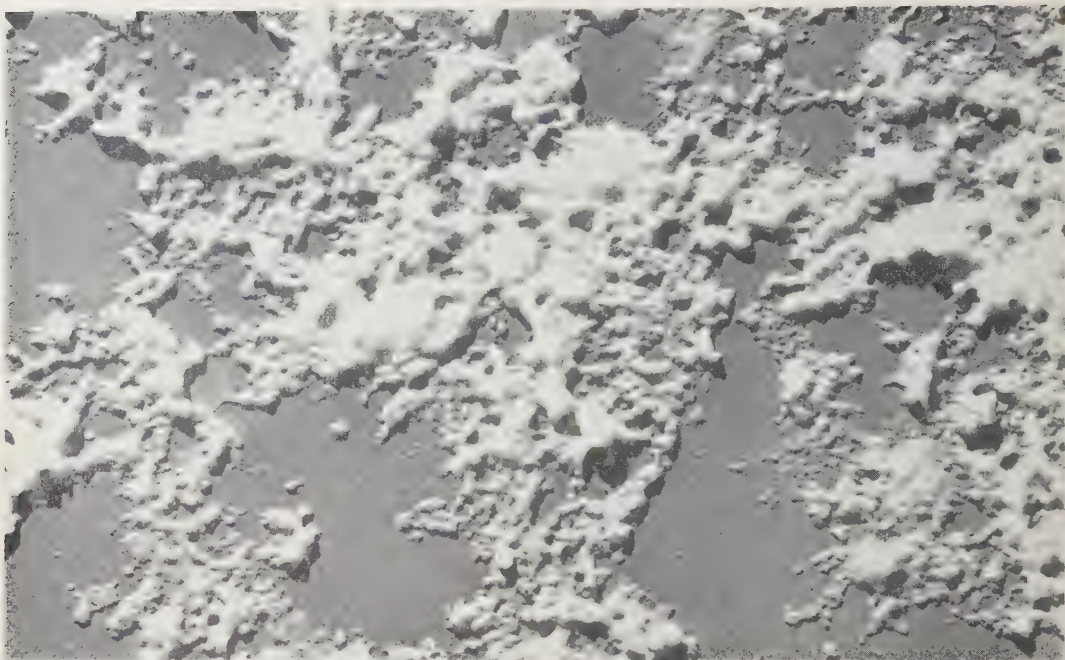


FIG. 3. Filtered protoplasmic fraction from ether-killed yeastlike cells of *H. capsulatum*. Magnification 33000 \times .



FIG. 4. Unfiltered protoplasmic fraction from merthiolate-killed cells of yeast phase of *H. capsulatum*. Magnification 29000 \times .

cells against human or experimentally infected animal sera was approximately 0.5 mg/ml, that for cell walls 0.125 mg/ml, and that for filtered protoplasm 4 to 8 mg/ml.

The possibility existed that some active antigen was washed out of the cell wall into the supernatant liquid in the process of separating the protoplasm. Accordingly, both the sediment and the supernatant fluid were assayed as complement-fixing antigens before initial washing in distilled water, and individually after each successful washing by centrifugation. The results indicated there was a marked increase in complement-fixing powers in both sediment and supernatant liquid as the cell walls were washed free of adherent protoplasm. The increase in activity of the cell-wall fraction may be attributed to gradual separation of the inactive protoplasm from the antigenically active cell wall. The increase in activity of protoplasm was attributed to leaching out of the active complement-fixing antigens from the cell wall during the washing process.

Intraperitoneal injection of the filtered soluble protoplasm or of the cell wall from merthiolate-killed or ether-killed cells induced the formation of complement-fixing antibodies in normal guinea pigs and rabbits, when heat-killed whole cells of the yeast phase were used as complement-fixing antigen. However, cell walls proved to be a much stronger antigen (Table I). When another group of rabbits was similarly injected and subsequently infected with an intravenous dose of 10^7 live cells of *H. capsulatum*, a much greater booster response was noted in the cell-wall fraction as compared with either whole cells or the soluble supernatant liquid (Fig. 5 a and b).

Further exposure of the cell wall to vibration in the Mickle disintegrator resulted in a suspension of minute fragments of cell wall. This suspension, which was clear to the naked eye, was very active antigenically in fixing complement in the presence of sera from cases of human histoplasmosis.

Protection. Mice were protected against lethal intracerebral or intraperitoneal challenge by prior injection of formalin-killed acetone-dried cells of the yeast phase, administered in one or 2 doses. When a single dose

TABLE I. Complement-Fixation Titers of Sera from 40 Rabbits Injected with 30 mg of Cell Walls, Protoplasm, or Whole Cells of *H. capsulatum*.*

	Protoplasm	Cell walls	Whole cells	Controls
Range	0-16	128-2048	16-64	0
Median	4	256	32	0
Mode	0	256-512	32	0

* Heat-killed whole cells of the yeast phase were used as the complement-fixing antigen. Ten rabbits were included in each of 4 groups.

was used, 5 mg were administered intraperitoneally and 2 weeks later the animals were challenged. When 2 immunizing doses were employed, $2\frac{1}{2}$ mg injections were given one week apart, and one week after the second dose the mice were challenged. Five mg of either cell-wall material or filtered protoplasm were injected intraperitoneally and the mice subsequently challenged intracerebrally with a lethal dose. In all cases, mice immunized with whole cells and non-immunized mice were included as controls. The protoplasm showed little or no protective powers in contrast to cell-wall material which did have good protective properties. Thus, only 22 of 69 mice (32%) died when first injected with cell walls, and subsequently challenged, in contrast to 53 of 60 mice (89%) that died when first inoculated with the protoplasm and subsequently challenged. Only 32 of 90 mice (35%) immunized with whole cells succumbed to the lethal challenge, while 87 of 93 (93%) of controls died. The cell wall was effective in quantities of approximately 0.5 to 5.0 mg of dried material per mouse, while the optimum dose for whole cells was approximately 5 mg per mouse.

Toxin. The toxic action of protoplasm and cell walls was examined in mice, with 2 mg of dried tubercle bacilli incorporated into each mouse dose. When 5 mg or 10 mg of antigen were injected per mouse (Table II), most of the deaths within 48 hours occurred as a result of the action of the cell walls and not of the soluble protoplasm. None of 160 mice receiving adjuvant alone died.

Hypersensitivity. Mice were inoculated intraperitoneally with sublethal dose of 10^6 72 hour cells of the yeast phase. Fourteen days later, these mice were challenged with equal

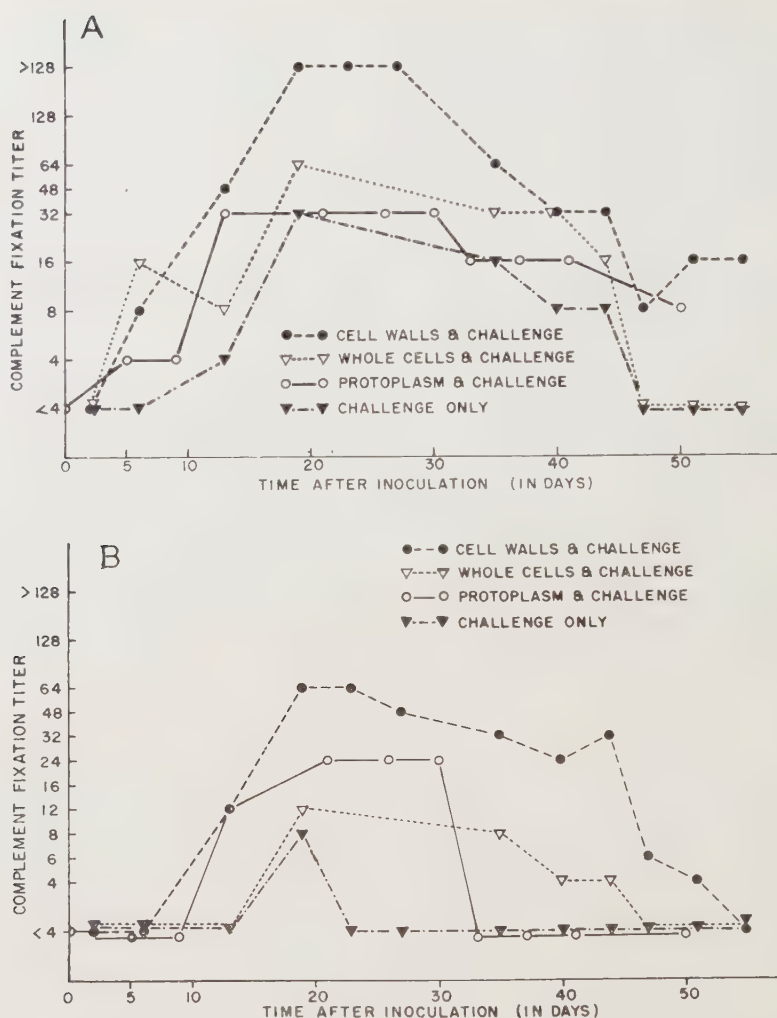


FIG. 5. Influence of cell walls or protoplasm on development of complement-fixing antibodies in experimentally infected rabbits. A. Peak titers. B. Median titers.

weights of whole cells, cell walls, or protoplasm, suspended in 5% mucin. Death rates were high and similar in mice that had been inoculated with cell-wall material or protoplasm during 48 hours post challenge. Either of these fractions produced a higher mortality than whole cells. For example, when 7.5 mg in 5% mucin were injected intraperitoneally per mouse, 40 of 50 mice (80%) succumbed to cell walls, 31 of 35 (89%) to protoplasm, and 12 of 50 (24%) to whole cells. Five of 148 mice (3%) sensitized but not further inoculated, died during this period. None of 50 sensitized mice succumbed after intraperi-

toneal inoculation of 5% mucin alone. Antigen, or antigens, active in initiating a response in sensitized mice seems to be present in both cell wall and protoplasm. Thirty-five guinea pigs which had been infected with a sublethal dose of the yeast phase were subsequently skin tested with the fractions previously described, as well as with histoplasmin. Edema developed 24 to 48 hours after intradermal injection, both with the cell-wall fraction and with filtered protoplasm. When the fractions were diluted to a similar extent, a slightly greater response was initiated by the soluble protoplasm. Five uninfected guinea pigs showed

TABLE II. Comparison of Toxic Action of Protoplasm and Cell Walls in 21-Day-Old Male Mice.

	Proto- plasm	Mortality		
		Cell walls	Whole cells	Adjuvant controls
5 mg	2/70* (3%)	11/70 (16%)	3/70 (4%)	0/70 (0%)
10	10/90 (11%)	55/90 (61%)	16/90 (18%)	0/90 (0%)

* Numerator represents No. of mice that died; denominator represents total No. of mice inoculated. These figures are totals from 3 separate experiments.

no skin reactions at all. Results in sensitized guinea pigs were, therefore, similar to those obtained in sensitized mice.

Hemolysis. A 0.5% suspension of guinea pig erythrocytes in 0.85% saline underwent hemolysis when exposed to 0.125 to 0.25 mg whole cells/ml. Protoplasm showed about as much activity over a similar weight range. The cell wall in comparison showed little hemolytic activity with concentration as high as 8 mg/ml not producing complete hemolysis.

Discussion. With the methods of preparation used in the foregoing experiments, the cell wall of the yeastlike cells of *H. capsulatum* contained most of the antigenic activity responsible for the fixation of complement in the presence of specific antiserum. This fixation of complement occurred with human sera from proven or suspected cases of histoplasmosis and with animal sera from experimentally infected rabbits or guinea pigs. The antigen associated with complement fixation is soluble in water, since extensive washing caused it to pass gradually into the supernatant liquid. Attempts, therefore, to prepare a soluble, purified, specific antigen from the yeast-like cells should be directed toward the cell wall.

The cell wall can be readily separated from the internal protoplasm by vibration and subsequent centrifugation. Mechanical agitation with the Mickle disintegrator apparently cracked the cell walls and released the protoplasm in a dispersed state. The products of disruption, cell walls and protoplasm, were then readily separated by centrifugation. The amount and type of vibration should be carefully controlled, in combination with electron-

microscope examinations, in order to permit ready separation of the two fractions. If the cells are exposed to excessive vibration or grinding, then the resulting cell-wall fragments are too small to separate from the coarse granular protoplasm. Cell disintegrators like the Raytheon ultra-sonic oscillator were accordingly found not to be useful, since they tended to fragment the cell wall excessively rather than merely to crack it (19), and, therefore, to make impossible the recovery of purified cell-wall fractions. Excessive ether or merthiolate treatment tended to coagulate the protoplasm and prevent its release in a dispersed state, to such an extent that separation of the protoplasm by washing and centrifugation became impossible.

Similarly, most of the activity responsible for the protection of mice or the toxic death of mice was situated in the cell wall. The identity of or relationship between these 3 antigenic actions is still in doubt. It is of interest, however, to note that complement-fixing antibodies were not readily detected in immunized mice which had marked resistance to reinfection or to lethal challenge. Rabbits and guinea pigs, on the other hand, readily developed complement-fixing antibodies in high titers, but showed little tendency to develop marked resistance to lethal challenge (19).

A delayed reaction in sensitized animals was produced by both cell walls and protoplasm. This result may mean that parts of either fraction are capable of initiating a response, or that the particular antigen involved is highly soluble and readily dissolves out of the cell wall into the surrounding fluid.

Since the tissue phase of *H. capsulatum* is yeastlike in some of its properties, the possibility has presented itself that zymosan may be present in the cell wall (20). If zymosan is present, then it would tend to reduce the bactericidal, and possibly mycoidal, properties of serum. However, the cell walls of *H. capsulatum* make the mice more resistant to subsequent challenge, rather than less resistant. Hence, the zymosan-properdin system, if present, has no obvious influence on the immunization of mice against *H. capsulatum*.

Summary. A technic, involving the use of

the Mickle tissue disintegrator and the electron microscope, was described for the separation of the cell wall and the internal protoplasm from the cells of the yeast phase of *Histoplasma capsulatum*. The cell wall contained the antigenic activity associated with complement fixation, protection and toxins. Sensitized animals, on the other hand, showed a delayed type of reaction to either fraction. The protoplasm was more active than the cell wall in the hemolysis of guinea pig erythrocytes.

1. Salvin, S. B., PROC. SOC. EXP. BIOL. AND MED., 1947, v66, 342.
2. Grayston, J. T., *J. Lab. and Clin. Med.*, 1952, v40, 90.
3. Saslaw, S., and Campbell, C. C., *ibid.*, 1948, v33, 1207.
4. Salvin, S. B., and Hottle, G. A., *J. Immunol.*, 1948, v60, 57.
5. Salvin, S. B., *J. Lab. and Clin. Med.*, 1949, v34, 1096.
6. ———, *J. Immunol.*, 1950, v65, 617.
7. Campbell, C. C., and Binkley, G. E., *J. Lab. and*

Clin. Med., 1954, v42, 896.

8. Campbell, C. C., *Am. J. Pub. Health*, 1953, v43, 712.
9. Sorenson, L. J., and Evans, E. E., PROC. SOC. EXP. BIOL. AND MED., 1954, v87, 339.
10. Salvin, S. B., *J. Immunol.*, 1952, v69, 89.
11. ———, *ibid.*, 1953, v70, 267.
12. ———, *Am. J. Hyg.*, 1955, v61, 72.
13. Zarafonitis, C. J. D., and Lindberg, R. B., *Univ. Hosp. Bull.*, Ann Arbor, 1941, v7, 47.
14. Salvin, S. B., *J. Immunol.*, 1955, in press.
15. ———, PROC. SOC. EXP. BIOL. AND MED., 1951, v76, 852.
16. Skinner, C. E., Emmons, C. W., and Tsuchiya, H. M., *Molds, Yeasts, and Actinomycetes*. John Wiley and Sons, N. Y., 1947.
17. Ribí, E., and Salvin, S. B., *Experimental Cell Research* (in press).
18. Salvin, S. B., and Furcolow, M. L., *J. Lab. and Clin. Med.*, 1954, v43, 259.
19. Salvin, S. B., and Ribí, E., unpublished results.
20. Pillemer, L., Blum, L., Lepow, I. H., Ross, O. A., Todd, E. W., and Wardlaw, A. C., *Science*, 1954, v120, 279.

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Reproduction in Rats Fed *Lathyrus* Peas or Aminonitriles. (22013)

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Growth disturbances produced by feeding *Lathyrus* peas vary inversely with age and stage of development of the experimental animal(1,2). Profound effects might be expected, therefore, during rapid growth and development of the fetus. Turney, Salmon, and Copeland reported that feeding *Lathyrus hirsutus* peas prevented completion of pregnancy in the rat, but they did not completely describe the nature of the disturbance(3). They were unable to prevent the effect of peas by addition of alpha-tocopherol to the diet(4), although it has since been reported that large doses of that compound give partial protection against lathyrism(5). For these reasons the effect of *Lathyrus odoratus* peas was studied in regard to reproduction. During this investigation, isolation of the active principle of *Lathyrus pusillus* was announced(6). A sim-

ilar isolation product of *Lathyrus odoratus* was identified as (β -L-glutamyl-amino) propionitrile(7). The characteristic activity in producing skeletal lesions in rats was also shown by β -amino-propionitrile(1). This compound also profoundly influenced development of larval frogs(2). Aminoacetonitrile was later found to be more toxic to weanling rats than any compound previously tested(8). A number of aminonitrile compounds were consequently included in this study.

Methods. Albino rats of Sprague-Dawley strain were used predominantly. The few hooded rats of Long-Evans strain used gave identical results. The basic diet was ground (Rockland) rat pellets. *Lathyrus odoratus* seeds were finely ground, mixed with this diet in various proportions, refrigerated at 5°C, and fed *ad libitum*. Diets containing syn-

thetic nitrile compounds were stored at -40°F . Feedings were given once daily, and food remaining after 24 hours discarded. All animals were kept on wire-mesh floors. The 50% pea diet produced skeletal deformities and fatal aortic rupture comparable to that reported by Ponseti and Baird(9). Females were first mated when 10-12 weeks of age, and discarded after 3 or 4 matings. Matings were determined by vaginal smear. Specimens for histological examination were fixed in 4% formalin and stained with hematoxylin and eosin.

Results. Mature rats of both sexes maintained on 50% pea diet remained in good health for several months. Two male rats placed on this regimen at 6 weeks of age developed extensive skeletal deformities within a few weeks, but accomplished numerous fertile matings between 3 and 9 months. When sacrificed at 10 months they were moderately emaciated and almost helplessly crippled, but showed active spermatogenesis, without testicular atrophy. A third male, started on 50% pea diet at 3 months, was moderately crippled but still potent and fertile when sacrificed 10 months later. This male also showed histologically normal testes. The numerous offspring obtained by mating these males with normal females showed no abnormalities.

The 50% pea diet given to nursing female rats produced no evident toxic effect. In one case this diet was given to the dam of 6 young, beginning on the 3rd day after parturition. In 2 instances this diet was begun on the 20th day of pregnancy, and continued until litters were weaned. Large litters were born, of which 3 in one litter and 5 in the other survived the early neo-natal period. The high peri-natal mortality was typical of results obtained when pea diets were given late in pregnancy, and will be discussed in detail later. The nurslings of all 3 litters made normal weight gains, with no physical or x-ray evidence of skeletal deformity at weaning at 22 days. Three males and 3 females given 50% pea diet after weaning quickly developed severe skeletal deformities, and 2 animals of each sex died of ruptured aortic aneurisms within a month. Those given control diet grew to normal maturity.

Mature female rats on 50% pea diet passed

TABLE I. Effect of 50% Pea Meal.

No. rats	Days of gestation diet given	Avg No. viable young
9	1-22	0
4	1-16	8.5
6	1-17	.67
4	16-18	.25
6	19-22	0
7	20-22	4
4	21-22	12

through normal estrous cycles, and mated freely. Conception occurred in a high percentage of matings, with bloody vaginal "placental sign" on about the 13th day. Weight gains were comparable to control animals until the 19th or 20th day, but then weight rapidly decreased, and no living young were born. Pregnant females sacrificed on or before 19th day of pregnancy contained living fetuses, but during the succeeding 3 days there was death and rapid decomposition of all unborn young. Passage of dead fetuses or placental tissue was seldom observed, although vaginal bleeding was often seen at the 22nd or 23rd day. Animals killed within a few days after this time showed involuting uteri with numerous placental sites. Those kept on 50% pea meal soon displayed normal estrous cycles culminating in fertile matings. If the control diet was then given, normal gestations resulted.

The final few days of gestation constituted the critical period for exhibition of the toxic effect of the pea (Table I). Normal litters were born to females on 50% pea diet during the first 16 days of pregnancy, but giving this diet for one more day resulted in death of almost all fetuses. Giving the diet for as short a time as 3 days after the 16th day was also highly toxic for the unborn young.

Diets containing 20% *Lathyrus odoratus* meal were lethal for the rat fetus, if begun by the 17th day of gestation (Table II). The

TABLE II. Percentage of Lathyrus Pea Meal Begun 17th Day of Gestation.

No. rats	% pea meal	Avg No. viable young
9	50	0
8	20	.5
2	15	2
3	10	10

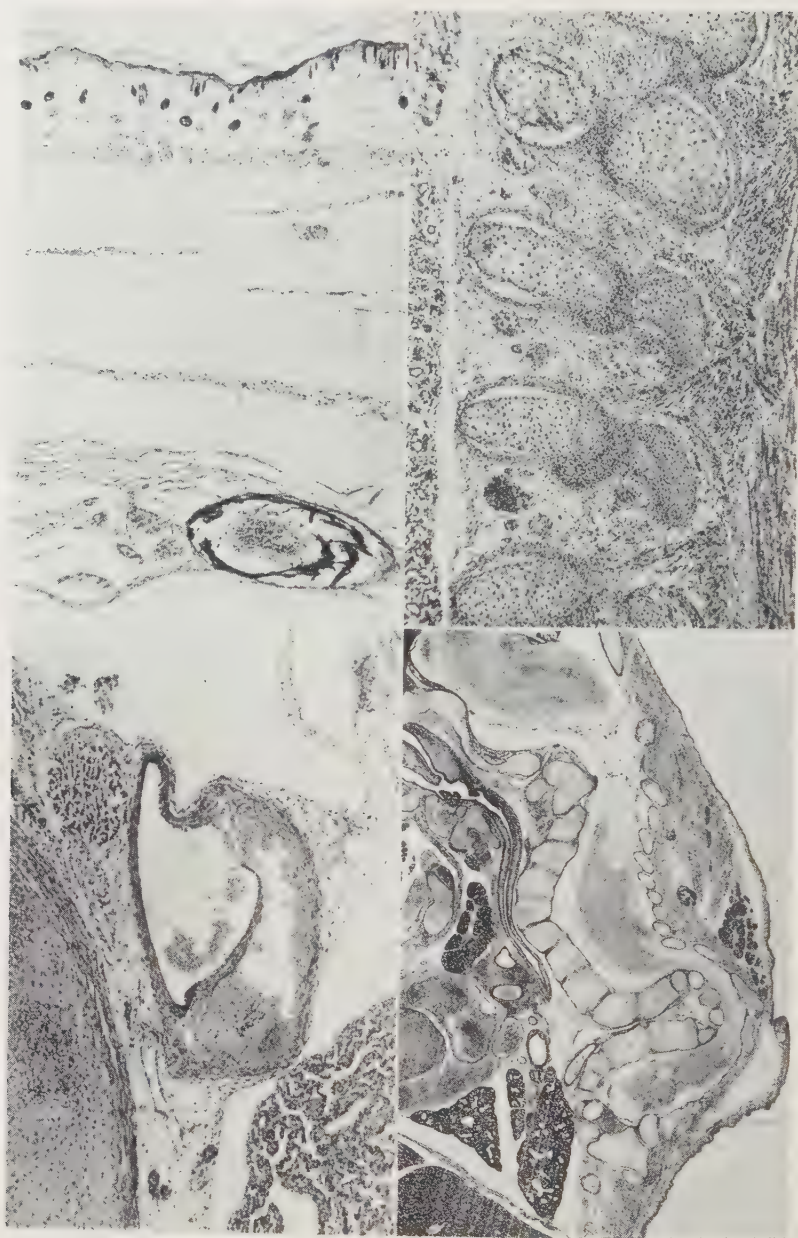


FIG. 1 (top left). Microphotograph, posterior thorax of still-born rat. Includes tissues from skin surface to parietal pleura, with cross section of rib, lower right. There is extreme generalized edema, with paucity of connective tissue and skeletal muscle.

FIG. 2 (top right). Microphotograph, parasagittal section, thoracic spine of 19-day rat fetus, alive at time of sacrifice of dam, who had received 50% pea diet from first day of pregnancy. Note lack of cohesion between cartilaginous spinal structures and perichondrial connective tissue.

FIG. 3 (bottom left). Microphotograph, cross section of dissecting aneurysm, thoracic aorta of still-born rat whose dam received 50% pea diet from 17th day of pregnancy. The intima is ruptured and a large mass of blood has forced apart the layers of the media. Complete rupture of the vessel, with massive hemothorax, has not taken place in this case.

FIG. 4 (bottom right). Microphotograph, mid-sagittal section, 19-day rat fetus, litter-mate of fetus shown in Fig. 2. There is acute angulation of the lower thoracic and lumbar spine, with apparent compression of the spinal cord. Diffuse hemorrhage is seen, particularly about great vessels at base of heart. The fetus was alive at time of sacrifice of the dam.

TABLE III. Toxicity of Aminonitriles Begun 17th Day of Gestation.

Compound	% in diet	No. rats	Avg No. viable young
Aminoacetonitrile*	.0167	4	0
	.0125	10	3.1
β -aminopropionitrile	.3	3	0
	.1	9	0
	.05	6	0
	.025	9	6.2
Bis (β -cyanoethyl)amine	.1	3	0
	.05	2	10
Tris (β -cyanoethyl)amine	.3	3	8
β -dimethylaminopropionitrile	.3	2	9
β -mercaptoethylamine	.5	5	3.4
	.25	3	5.7

* Fed as a salt of H_2SO_4 .

15% level was incompletely lethal, and lesser concentrations apparently harmless. At 20% level many young were born dead, with little evidence of decomposition, while others were feebly alive at birth, but succumbed soon thereafter. Both living and dead were fragile and hydropic in character (Fig. 1). Muscles and connective tissues were poorly developed. There was scanty formation of collagen, and cohesion of various skeletal and connective tissues appeared faulty (Fig. 2). Poor vascular tone, with capillo-venous dilatation and diffuse hemorrhage was commonly seen. Massive hemothorax from aortic rupture was a frequent demonstrable cause of death (Fig. 3). The dissecting aortic aneurisms followed the same pattern seen in weanling rats(1,9). Severe spinal deformities seen in several instances included kypho-scoliosis similar to those typically produced in weanling rats on *Lathyrus* pea diets (Fig. 4).

Several nitrile compounds were toxic for the rat fetus (Table III). Their effect appeared to be qualitatively identical with that of pea diets. Fetal death was readily produced by feeding diets containing β -aminopropionitrile, bis (β -cyanoethyl) amine, or aminoacetonitrile during the latter part of pregnancy. Aminoacetonitrile was the most toxic of these compounds, as shown by Wawzonek and co-workers(8). Lesions produced by these compounds mimicked those produced by pea meal diets, including a high incidence of aortic rupture with hemothorax. As with pea meal diets,

little toxicity was shown during the first 16 days of gestation. Thus, normal litters were born to female rats fed a diet containing 0.1% β -aminopropionitrile during the first 16 days of pregnancy, whereas diets with 0.05% of this compound proved uniformly lethal to fetuses when given beginning the 17th day of pregnancy (Table III). It is perhaps significant that the obvious developmental defects produced involved mesodermal tissues, and that development of these lesions occurred during the period of rapid mesodermal growth and differentiation of late fetal life. A possible difference in placental permeability in regard to the toxic compounds might also account for differences in toxicity shown during early and late fetal life.

Two nitrile compounds tested, tris (β -cyanoethyl) amine and β -dimethylaminopropionitrile, showed no toxicity at concentrations used. These results are in accord with previous reports of the detoxifying effect of methylation or acetylation of aminonitrile compounds(10). The final compound listed in Table III, β -mercaptoethylamine, while not an aminonitrile, has produced skeletal growth disturbances typical of lathyrism(11). This compound was found to be relatively innocuous for the pregnant rat and her unborn young, although at comparatively high concentrations many fetuses did die *in utero*. None of these fetuses, however, showed skeletal deformity, ruptured aortae, or other lesions characteristic of lathyrism.

Summary. *Lathyrus odoratus* peas and certain aminonitrile compounds chemically related to the active extract of the pea, when added to the diet of pregnant rats, caused death of the fetal rat late in intrauterine life. Death was associated with poor development of the skeleton and other mesodermal tissues, and commonly resulted from rupture of the thoracic aorta at or near the time of birth. No harmful effects on the fetus were demonstrated by feeding these substances to the pregnant female before the 17th day of gestation. *Lathyrus odoratus* peas in 50% dietary concentration did not affect fertility of the adult male or female rat, and males maintained on the diet for as long as 10 months showed no histological evidence of testicular atrophy.

No toxic substance was demonstrated in the milk of nursing females on the pea diet. The feeding procedure employing pregnant female rats proved to be a reasonably accurate and sensitive method for the toxicity assay of aminonitriles. Aminoacetonitrile was the most toxic of the compounds tested.

1. Ponseti, I. V., and Shepard, R. S., *J. Bone and Joint Surg.*, 1954, v36-A, 1031.

2. Chang, C. Y., Witschi, E., and Ponseti, I. V., *Anat. Rec.*, 1954, v120, 816.

3. Turney, D. M., Copeland, D. H., and Salmon, W. D., *Ala. Agri. Exper. Sta. Ann. Rep.*, 1943-44, v54-55, 18.

4. Turney, D. M., Salmon, W. D., and Copeland, D. H., *ibid.*, 1945-46, v56-57, 18.

5. Lee, J. G., *J. Nutrition*, 1950, v40, 587.

6. Dupuy, H. P., and Lee, J. G., *J. Am. Pharm. A*, (Scient. Ed.), 1954, v43, 61.

7. Schilling, E. D., and Strong, F. M., *J. Am. Chem. Soc.*, 1954, v76, 2848.

8. Wawzonek, S., Ponseti, I. V., Shepard, R. C., and Wiedenman, L. G., *Science*, 1955, v121, 63.

9. Ponseti, I. V., and Baird, W. A., *Am. J. Path.*, 1952, v28, 1059.

10. Bachhuber, T. E., Lalich, J. J., Angevine, D. M., Schilling, E. D., and Strong, F. M., *Proc. Soc. Exp. Biol. and Med.*, 1955, v89, 294.

11. Dasler, W., *ibid.*, 1955, v88, 196.

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Effect of Diet on Betaine-Homocysteine Transmethylase of Rat Liver.* III. B-Vitamins Other Than Vitamin B₁₂. (22014)

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In the rat, a dietary deficiency of vit. B₁₂ causes a decrease in the betaine-homocysteine transmethylase activity of the liver(1-4). The addition of a purified "coenzyme" preparation of the transmethylase, to liver homogenates prepared from rats fed a diet deficient in vit. B₁₂, has recently been shown to restore the transmethylase activity(4). To ascertain whether this effect is specific for vit. B₁₂ the effect of a deficiency of each of the other B-vitamins on the transmethylase activity of rat liver has been investigated.

Methods. Albino rats of the Sprague-Dawley strain were used throughout the investigation. The rats were kept in individual cages and were fed *ad libitum*, unless otherwise stated. The complete vitamin mixture used for the various basal diets described below

provided in mg per 100 g of ration: thiamine hydrochloride, 0.5; riboflavin, 0.5; niacin, 1; calcium pantothenate, 2; pyridoxine, 0.25; folic acid, 0.02; biotin, 0.01; vit. B₁₂, 0.002; inositol, 10; and choline chloride, 150. Each week each rat was given orally 2 drops of halibut liver oil fortified with vit. E and K (5). For the deficient diets the vitamin under study was omitted and in some cases succinyl sulfathiazole ("sulfasuxidine") or the corresponding antivitamin was added to the diet as described in the tables of results. All diets contained 4% of salts 4(6) and 5% of corn oil. The basal diets contained the following percentages of protein, sucrose and supplementary amino acids: Diet I. Casein 25, sucrose 65.8. Diet II. Casein 25, DL-methionine 0.2, sucrose 65.6. Diet III. Casein 12, DL-methionine 0.2, DL-threonine 0.36, sucrose 78.2. Diet IV. Egg white 24, sucrose 66.8, the biotin content of the complete diet was increased to 0.1 mg per 100 g of ration. Diet V. Casein 9, gelatin 12, sucrose 69.8.

At the end of the experimental period the rats were killed by decapitation and were bled, and their livers were removed immediately and chilled in ice. Pooled homogenates

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TABLE I. Effect of Vitamin Deficiencies on Activity of Betaine-Homocysteine Transmethylase of Rat Liver.

Vitamin	Diet [*]	Exp. I			Duration of exp., days	Diet [*]	Exp. II		
		Control, g/wk	Rate of gain, g/wk	Transmethylase Control, μ g			Control, g/wk	Rate of gain, g/wk	Transmethylase Control, μ g
Thiamine	I	41.3	9.8	410	10	II	33.6	24.5	445
Riboflavin	I	35.0	19.5	320	10	II	33.6	24.2	445
Niacin	I	38.8	37.1	360	9	III	23.3	10.1	400
Pyridoxine	I†	52.0	5.0	405	10	II	33.6	14.0	445
Pantothenic acid	I	38.8	17.8	220	10	II	33.6	24.5	445
Biotin	I†	40.0	35.0	360	24	IV	30.1	23.3	490
Choline	V‡	21.0	15.0	575	9	V	21.0	9.1	385

* Diets are described in the experimental section. Weanling rats were used throughout unless otherwise noted.

† This diet contained 3 mg % desoxypyridoxine. Initial wt of rats was approximately 85 g.

‡ This diet contained 2% sulfasuxidine.

§ Initial wt of rats was approximately 150 g.

were prepared from the livers of 5 or 6 rats from each experimental group and the transmethylase activity was determined as described previously(4). The transmethylase activity is expressed as μ g of methionine formed per g of fresh liver during a 3 hour incubation period.

Results. The effect of a deficiency of each of the following: thiamine, riboflavin, niacin, pyridoxine, pantothenic acid, biotin and choline, on the activity of the betaine-homocysteine transmethylase of rat liver is shown in Table I. It can be seen that the transmethylase activity of the livers of the rats fed the deficient diets was generally *higher* than that of the control group. Only in the case of a biotin (Exp. II) or a choline deficiency was the transmethylase activity found to be lower than that of the corresponding control group. The effect of the biotin deficiency on the transmethylase was evident only when the deficiency was severe enough to retard the growth of the rats significantly (compare rate of gain and transmethylase activity in Exp. I and II) and even then the absolute value for the deficient group was quite high. Omission of choline from the (high protein) diet used in these experiments lowered the transmethylase activity only slightly; however, the results are in agreement with previous findings with low protein diets(7).

The results of 5 experiments with rats made deficient in folic acid in various ways are given in Table II. The transmethylase activity of livers of deficient rats was in each case higher than in the corresponding control group. This is particularly noticeable in experiments in which no antivitamin (Aminopterin, Lederle Laboratories, Pearl River, N. Y.) was administered. Experiments with paired rats (Exp. II) demonstrated that high transmethylase activity of deficient animals could not be attributed to an effect of starvation(7). Livers from rats fed diet deficient in both folic acid and vit. B₁₂ (Exp. III) also had a higher transmethylase activity than livers of rats fed the complete diet. As the diet in this case contained casein, and growth was retarded by development of a folic acid deficiency, it is doubtful whether a deficiency of vit. B₁₂ developed. Dinning *et al.*(8) have

TABLE II. Effect of Folic Acid Deficiency on Activity of Betaine-Homocysteine Transmethylase of Rat Liver.

Exp. No.	Treatment	Duration of exp., days	Initial wt of rats, g	Rate of gain		Transmethylase	
				Control, g/wk	Deficient, g/wk	Control, μ g	Deficient, μ g
1	Diet I + 2% sulfasuxidine	21	45	40.0	25.0	360	685
2	" I + 2% sulfa (pair fed)	21	45	25.2	18.5	380	800
3	" I + 2% sulfa—B ₁₂	21	45	35.6	21.0	365	840
4	" I + 4 mg/kg aminopterin	7	150	-23.0	-23.0	445	505
5	" II + 1 " "	7	210	20.0	15.0	475	485

reported that, in chicks, a dietary deficiency of folic acid causes a decrease in betaine homocysteine transmethylase activity of the liver. Williams(9) found liver transmethylase activity of rats fed a complete diet containing aminopterin (4 mg/kg of diet) and showing very severe symptoms, to be lower than that of comparable group receiving complete diet without aminopterin. It is difficult to compare our results with these results since in our investigation, comparison was made between a group that received a complete diet and one that received a folic acid-deficient diet, both of which received aminopterin. It is of interest that Kring and Williams(10) found the activity of liver tyrosine oxidase system, higher in rats receiving diets deficient in thiamine than in control rats receiving an adequate diet, and that Beaten and Ozawa(11) found the activity of glutaminase I to be increased in vit. B₆-deficient rats. There are also several reports (see reference 10) that folic acid deficiency causes increased xanthine oxidase activity of rat liver.

A comparison of values for growth and for transmethylase activity of livers of rats fed Diet I (containing 25% casein) and Diet II (Diet I, supplemented with 0.2% DL-methionine) shows that a supplement of sulfur amino acids stimulated synthesis of betaine-homocysteine transmethylase although no growth response was observed.

The experiments do not provide any clue as to the nature of the "coenzyme" of betaine-homocysteine transmethylase. However, the data presented here and elsewhere(4,7), give

some insight into the extremely complex relationships that apparently exist between dietary factors and activity of this enzyme.

Summary. When a diet deficient in one of the following vitamins: thiamine, riboflavin, niacin, pyridoxine, pantothenic acid, or folic acid was fed to rats, the activity of the betaine-homocysteine transmethylase of their livers was found to be higher than that of the livers of rats fed a complete diet. Deficiencies of biotin or choline, on the other hand, caused a slight decrease in the transmethylase activity.

1. Oginsky, E. L., *Arch. Biochem.*, 1950, v26, 327.
2. Williams, J. N., Jr., Monson, W. J., Sreenivasan, A., Dietrich, L. S., Harper, A. E., and Elvehjem, C. A., *J. Biol. Chem.*, 1953, v202, 151.
3. Mistry, S. P., Vadapalaite, I., Chang, I., Firth, J., and Johnson, B. C., *ibid.*, 1955, v212, 713.
4. Ericson, L.-E., Harper, A. E., Williams, J. N., Jr., and Elvehjem, C. A., *ibid.*, in press.
5. Harper, A. E., Benton, D. A., Winje, M. E., Monson, W. J., and Elvehjem, C. A., *ibid.*, 1954, v209, 165.
6. Hegsted, D. M., Mills, R. C., Elvehjem, C. A., and Hart, E. B., *ibid.*, 1941, v138, 459.
7. Ericson, L.-E., and Harper, A. E., *ibid.*, in press.
8. Dinning, J. S., Keith, C. K., and Day, P. L., *ibid.*, 1951, v189, 515.
9. Williams, J. N., Jr., *Proc. Soc. Exp. Biol. and Med.*, 1951, v78, 206.
10. Kring, J. P., and Williams, J. N., Jr., *ibid.*, 1954, v87, 97.
11. Beaten, J. R., and Ozawa, G., *J. Biol. Chem.*, 1955, v214, 685

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Transmission of Street Rabies Virus Strain (V308) to Suckling Hamsters During Lactation. (22015)

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It has been assumed that a bitch infected with rabies transmitted the disease to the puppies by injuring them by biting or other mechanical injuries. There is still the question as to whether or not rabies virus may be present in the milk and if so whether the puppies can become infected from this source. Specific inclusion bodies called Negri bodies (1) are present in the central nervous system of animals dying with rabies. These are most frequently demonstrable within the nerve cells and are eosinophilic bodies, usually spherical, 1 to 30 μ in size. Their inner structure shows basophilic granules. The Syrian hamster infected with rabies virus shows Negri bodies in the brain in greater numbers and of larger size than those demonstrable in the Swiss albino mouse (2). The Syrian hamster was, therefore, selected for these experiments not only because of its Negri body "production" but because of ease in control and care.

Materials and methods. The virus for the present study was obtained from a dog brain sent to this laboratory for routine rabies examination. The hippocampus was removed aseptically, ground with alundum in a mortar, and diluted with physiological saline to a 10% suspension. Three-hundredths ml of this 10% suspension was injected intracerebrally into each of ten 3-week-old Swiss albino mice (Carworth Farm, Webster strain). On the 14th day after injection, several mice developed tremors and paralysis and became moribund. These brains were then removed aseptically. Impressions made from the Ammons horn of each mouse were stained with Seller's stain (3) and examination by the light optical microscope showed numerous Negri bodies throughout the tissue. The Ammons horn from one of these mice contained a larger number of Negri bodies than the others and it was ground with alundum and diluted to a 20% suspension with physiological saline. Each of ten 3-week-old Swiss albino mice was injected intracerebrally with this rich virus

suspension. Titer of the virus was $10^{-3.5}$ when titrated intracerebrally in Swiss albino mice of the same age. The 10 Swiss albino mice which were infected intracerebrally showed rabies symptoms within 6 to 10 days. Their brains which were removed aseptically contained Negri bodies and were ground and diluted to a 10% suspension in physiological saline. This virus-bearing mouse brain suspension was used to initiate the present study. For this series of experiments, diagnosis of rabies in hamsters and mice was made by the following criteria: (1) clinical observations of each hamster, (2) demonstration of Negri bodies in the Ammons horn of the suckling hamster, adult hamster and Swiss albino mouse, (3) reinoculation of the positive and negative suckling hamster brain and uninoculated controls intracerebrally into 3-week-old Swiss albino mice and confirmation by demonstration of Negri bodies in the brain smears. The presence of Negri bodies was demonstrated microscopically in touch preparations of the Ammons horn stained with Seller's stain. Ten lactating hamsters and their sucklings were placed in individual metal boxes with bedding. The sucklings were about 12 days old and are usually weaned at 16 to 18 days. Therefore, these were removed from the parent 6 days after her inoculation. The lactating hamster from each group (#1 to #9) was given 0.1 ml of the above described mouse brain bearing virus via the flank muscle. The area of injection was sterilized with iodine for one hour before the mother from each of the 9 groups was placed with her sucklings. The No. 10 group was kept as a room control. During the next 6 days, the family of each group was not disturbed. Dog checkers were fed to each of the 10 groups daily along with a large bottle of water for drinking (Table I). At the end of the sixth day, after inoculation of the parent, the sucklings from each group were weaned and placed in special glass jars. The lactating hamsters showed no rabies

symptoms at this time and were held for further observation. Seven of the 9 inoculated lactating adults developed rabies symptoms between the 14th and 22nd day after inoculation. Their brains contained numerous Negri bodies. The 10 groups of weaned hamsters from the 9 infected lactating animals and the control hamster were fed, watered and checked twice daily (Table II) for rabies symptoms.

Results. Two days after weaning or 8 days after injection of the adults, several hamsters from Groups 1, 2 and 3 (Table II) showed rabies symptoms and their brains contained numerous Negri bodies. Brains from all suckling hamsters showing rabid symptoms were ground in a mortar with physiological saline to a 20% suspension and injected intracerebrally into each of 6 Swiss albino mice. Mice showing rabies symptoms were examined for Negri bodies as shown in Table II. At the end of 21 days all suckling hamsters showing no rabies symptoms were sacrificed and their brains were removed aseptically and pooled for each group, ground in a mortar with alundum and diluted to a 20% suspension with physiological saline. Each of the 10 pooled groups, including the control Group #10, were injected (0.03 ml each mouse) intracerebrally in each of 6 Swiss albino mice (Table II) in order to confirm the presence or absence of the virus in sucklings showing no rabies symptoms.

Summary. From this observation it appears that rabies may be transmitted from lactating hamsters to suckling hamsters. Seventy-seven percent of the mothers showed rabies symptoms between 14 to 20 days.

TABLE I. Response of Lactating Hamsters Injected in Flank with 0.1 cc Street Virus Rabies Strain, V308. Suckling removed after 6 exposures. 9 groups.

Appearance of symptoms (days)	Negri bodies
14	+
16	+
16	+
16	+
17	+
20	+
20	+
—	—
—	—

TABLE II. Response of Suckling Hamster after Weaning from Lactating Parent (Infected with Rabies Street Virus V308).

Group No.	No. of suckling	Onset of rabies (days)	Negri bodies (sucklings)	Mouse inoculation	Negri bodies
1	5	8	+	+	+
		8	+	+	+
		9	+	+	+
		9	+	+	+
		9	+	+	+
2	5	9	+	+	+
		9	+	+	+
		9	+	+	+
		9	+	+	+
3	5	8	+	+	+
		8	+	+	+
		8	+	+	+
		10	+	+	+
		12	+	+	+
4	4	—	—	—	—
		—	—	—	—
5	4	22	+	+	+
		—	—	—	—
		—	—	—	—
6 to 9			Negative		
10	6		(uninoculated controls)		

* Showed no clinical symptoms.

31% of the suckling hamsters developed rabies symptoms between 8 to 22 days. Confirmation of rabies was made on all positive animals by Negri body examination. The possibility exists that rabies virus was present in the saliva of the mothers before the suckling animals were weaned, and consequently infection could have occurred by some route other than the milk. This could occur even without biting. Direct infection of mucous surfaces as the result of licking is possible, although this seems remote due to the high percentage of suckling hamsters showing rabies symptoms.

1. Negri, A., *Z. f. Hyg. u. Infektionskr.*, 1903, v43, 507.
2. Reagan, R. L., Day, W. C., and Brueckner, A. L., *PROC. SOC. EXP. BIOL. AND MED.*, 1952, v81, 654.
3. Sellers, V. F., *Am. J. Pub. Health*, 1929, v17, 1080.

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5-Hydroxytryptamine in Mast Cells.* (22016)

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Two important physiologically potent tissue constituents have been associated with mast cells: heparin by Holmgren and Wilander(1) and histamine by Riley and West(2). In the present communication, we shall provide evidence that 5-hydroxytryptamine is present in the mast cells of at least one species, the rat.

Mast cells were prepared from the peritoneal cavity of rats by a modification of the method of Padawar and Gordon(3). The cells of the final concentrate appeared well preserved; they maintained their characteristic staining with toluidine blue. There was evidence of some shedding of peripheral granules. Total and differential counts were performed using the solution designed by Moore and James(4) for blood basophiles. Mast cells constituted from 87 to 92%, usually 90 to 92%, of the total population in the final preparations. The remaining cells were mesothelial, mononuclear cells and a few polymorphonuclear leukocytes. Estimates of the volume of cells were made using a red blood cell diluting pipette of about 1 ml capacity centrifuged at $1000 \times g$ for 1.5 hours to constant volume. From 10 rats, 5 to 10 mm^3 of cells were obtained. The mean volume per cell was estimated in 4 determinations to be $1.26 \pm 0.13 \times 10^{-6} \text{ mm}^3$.

5-hydroxytryptamine was identified and estimated using the rat colon as described by Dalglish, Toh, and Work(5). The volume of the bath was 16 ml. For extraction of 5-hydroxytryptamine from the cells the final concentrate in sucrose, containing 1 to 1.4 mm^3 of cells per ml, was diluted 1:20 with distilled water and frozen and thawed 3 times. Although this does not grossly fragment the cells, it does release their histamine and 5-hydroxytryptamine.

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Identification of 5-hydroxytryptamine in the frozen and thawed preparation was made as follows: 1) The material contracted the rat colon in a manner identical with synthetic 5-hydroxytryptamine(6). The capacity of the material in the extract to contract the colon strip was abolished by repeated application of relatively large amounts, 1 to $2 \mu\text{g}$ of 5-hydroxytryptamine to the strip, as with 5-hydroxytryptamine itself(6). Lysergic acid diethylamide, $10 \mu\text{g}$ in the 16 ml bath for 10 minutes, abolished the reaction to both extracted material and synthetic serotonin. Dibenzamine hydrochloride, $100 \mu\text{g}$ in the bath, likewise abolished the effect of mast cell extract and reference standard(7). Final identification of the material was made by extraction and chromatography: 6.4 mm^3 of a preparation containing 92% mast cells was extracted 3 times with 95% acetone(6). The acetone was evaporated partly to dryness *in vacuo* at 32°C , applied to Whatman No. 1 filter paper and chromatogrammed using butanol-acetic acid-water (4:1:5)(5). The R_f value for the unknown was 0.44 and of synthetic standard was 0.46. Formaldehyde-bichromate spray and ultraviolet illumination were used for identification(8). The characteristic golden yellow fluorescence was observed.

The concentration of 5-hydroxytryptamine (as free base) was estimated by colon-assay to be $0.63 \mu\text{g}/\text{mm}^3$ of cells. From the chromatogram it was estimated to be about $0.7 \mu\text{g}/\text{mm}^3$. Evidence that the material is not present in the residual contaminating cells in the mast cell concentrate is as follows: In concentrating the exudate, the 5-hydroxytryptamine activity increases with increasing purity of mast cells. The skin and subcutaneous tissue of the rat contain material with identical activity on the rat colon strip and chromatographic mobility with the butanol-acetic acid-water solvent. The activity of this material

parallels the mast cell content in a manner similar to that previously shown for histamine(9).

The concentration of 5-hydroxytryptamine in the mast cells of the subcutaneous areolar tissue of the rat was computed from estimates of 5-hydroxytryptamine on homogenates of areolar tissue and measurements by the method of Chalkley(10) of the relative mast cell volume. By this means, a value of 0.72 μg 5-hydroxytryptamine per mm^3 of subcutaneous mast cells was obtained.

The evidence given demonstrates the presence of 5-hydroxytryptamine in the mast cells of the rat. We have found that this material has physiological activity when released by those agents which produce mast cell damage(11).

The presence and quantity of histamine and heparin in the isolated concentrated mast cells has been demonstrated using similar technics (12). The amount of histamine was estimated to be 10 $\mu\text{g}/\text{mm}^3$ and of heparin 30 $\mu\text{g}/\text{mm}^3$.

Definitive evidence of the presence of 5-hydroxytryptamine in mast cells along with histamine and heparin is important for several reasons: it emphasizes the significance of these cells as participants in the reaction to injury; it provides further evidence for a relationship between mast cells and blood platelets(13); finally, a connection is established between mast cells and the cells of the enterochromaffin system and with certain, as yet unidentified cells in the central nervous system since both of these, as well as the blood platelets, are now known to contain 5-hydroxytryptamine(14,6,15).

Summary. 5-Hydroxytryptamine as well as

histamine and heparin was demonstrated to be present in purified mast cells from the peritoneal washings of rats. The material was identified by bioassay and chromatography. The concentration of 5-hydroxytryptamine found in isolated mast cells agreed with that found by indirect means in the mast cells of the subcutaneous areolar tissue of rats. Implications of this finding are briefly discussed.

1. Holmgren, H., and Wilander, O., *T. Mikroskop.-Anat. Forsch.*, 1937, v42, 242.
2. Riley, J. F., and West, G. B., *J. Physiol.*, 1953, v120, 528.
3. Padawar, J., and Gordon, A. S., *PROC. SOC. EXP. BIOL. AND MED.*, 1955, v88, 29.
4. Moore, J. E., III, and James, G. W., III, *ibid.*, 1953, v82, 601.
5. Dalgliesh, C. E., Toh, C. C., and Work, T. S., *J. Physiol.*, 1953, v120, 298.
6. Amin, A. H., Crawford, T. B. B., and Gaddum, J. H., *ibid.*, 1953, v126, 596.
7. Gaddum, J. H., and Hameed, K. A., *B. J. Pharmacol.*, 1954, v9, 24.
8. Shepherd, D. M., West, G. B., and Erspamer, V., *Nature*, 1953, v172, 357.
9. Benditt, E. P., Bader, S., and Lam, K. B., *Arch. Path.*, 1955, v60, 104.
10. Chalkley, H. W., *J. Nat. Canc. Inst. (U.S.)* 1943, v4, 47.
11. Benditt, E. P., and Rowley, D. A., *Science*, in press.
12. Benditt, E. P., Lagunoff, D., and Wong, R., to be published.
13. Benditt, E. P., and Mitziga, D. W., *Proc. Chicago Inst. Med.*, 1955, v20, 308.
14. Tucker, M. B., and Rapport, M. M., *Fed. Proc.*, 1954, v13, 170.
15. Erspamer, V., *Rendicont Scientifici Farmitalia*, 1954, v1, 5.

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Species Differences in Serum Glycoproteins of Man, Dog and Rabbit, by Paper Electrophoresis.* (22017)

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Paper electrophoresis provides a simple technic for the identification of serum proteins and stainable components bound to serum proteins. By application of the periodic acid-Schiff method for histochemical identification of various carbohydrate substances, Köiw and Gronwall first adapted this technic for the study of serum glycoproteins(1). The purpose of the present study was to establish a quantitative pattern of these components and their distribution in relation to serum proteins in normal human serum and in plasma from dogs and rabbits.

Procedure. Paper electrophoresis was performed according to a method described elsewhere(2,3). Filter paper strips were run in parallel for subsequent determination of proteins and glycoproteins. For the latter, .040 cc of human serum and rabbit plasma were employed, and .060 cc of dog plasma. In certain instances a third strip was added for determination of lipoproteins (Oil-red-O stain). The staining procedure employed for glycoproteins differed from that of Köiw and Gronwall in several steps. In preparation of the fuchsin sulfite (Schiff's reagent) 15 to 20 ml of concentrated hydrochloric acid rather than dilute acid were added as a final step. The paper strips were immersed in this solution under constant shaking for time periods varying usually between 10-15 minutes. The staining was continued until clear visibility of the purple bands was obtained without coloring of the background. The densitometric evaluation of the strips was performed immediately afterwards because of their tendency to darken after drying(4). The original color of the strips can be restored by brief immersion in the "sulfite rinse." Individual determinations were performed on serum from 9 normal human beings, six men and three women, ages 15 to 55 years, and

clinically free of disease. Heparinized plasma from 6 normal male mongrel dogs and 8 normal male chinchilla rabbits were included in the study. The strips of human serum or animal plasma stained for glycoprotein (glycidograms) revealed 3 main components migrating with the mobility of alpha-1, alpha-2 and beta globulins. These appeared as distinct bands on the paper strips. The densitometric curves were readily separated into these 3 components. Gamma globulin was in our experience devoid of carbohydrate-staining material. Only faint amounts of material stained in the region of albumin; this fraction was therefore not included in evaluating the normal distribution pattern of the glycoproteins. Around the line of application of the serum a small amount of stainable material was usually visible which probably represents stainable substances adsorbed at the point of origin which showed no migration in the electrical field (0-fraction). Accordingly, the patterns were standardized only for the above-mentioned main components; alpha-1, alpha-2 and beta glycoproteins. Rough correlation was also observed in man between the total area of glycoprotein stained, measured by the planimeter, and the levels of total serum polysaccharide or hexosamine determined chemically.† This was noted despite the observation that different glycols may vary in their affinity for periodic acid(5,6). Even after filter paper strips had darkened on standing, densitometric evaluation after reimmersion in the "sulfite rinse" yielded curves and planimetric values identical to those obtained on initial examination. With strict adherence to this principle and with careful technic the method was found to be reproducible.

Results. In normal human serum the alpha-2 fraction was the largest (approximately

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TABLE I. Glycoprotein Distribution in Various Species.

Species	% of total stainable carbohydrate		
	Alpha-1	Alpha-2	Beta
Man	32.5	39.8	27.6
	24.6	41.1	34.2
	26.8	40.6	32.4
	33.9	39.7	26.4
	23.2	47.4	29.4
	31.7	35.0	33.3
	29.0	42.0	29.0
	31.4	42.9	25.7
	26.0	46.0	28.0
Mean	28.8 ± 3.8	41.6 ± 3.6	29.6 ± 3.1*
Dog	23.5	54.5	22.0
	27.0	52.5	20.5
	20.0	62.5	17.0
	21.0	52.5	26.5
	19.0	63.0	18.0
	29.0	51.5	19.5
Mean	23.2 ± 4.0	56.1 ± 5.3	20.6 ± 3.4*
Rabbit	92.5		7.5
	83.0		17.0
	85.0		15.0
	86.5		13.5
	90.0		10.0
	80.0		20.0
	82.0		18.0
	89.0		11.0
Mean	86.0 ± 4.3		14.0 ± 4.3*

* Statistical significance of differences of mean values for beta-glycoprotein: Man vs. dog, $p = .002$; man vs. rabbit, $p = <.0001$.

40%), while alpha-1 and beta were approximately equal, 30% each (Table I). In dogs, three glycoprotein bands were again identified; alpha-2 glycoprotein migrated somewhat more rapidly and was thereby closer in position to alpha-1 glycoprotein than in human serum (Fig. 1). The total quantity of stainable carbohydrate was less, necessitating the application of more plasma for a visible pattern (see procedure). Alpha-2 glycoprotein again was the main component, accounting, however, for even a greater percentage of the total stainable carbohydrate than in man (approximately 56%); alpha-1 and beta glycoprotein were present in about equal amounts but in lower proportion than in human serum (Table I). In rabbits, separation of alpha-1 and alpha-2 glycoprotein was not possible in many instances and, therefore, a single alpha-glycoprotein component was measured planimetrically. It was the major plasma glycoprotein component (approximately 86%) (Table I, Fig. 1). Beta glyco-

protein was in this species considerably lower than in the other two.

No alteration of the normal plasma glycoprotein pattern of the rabbit was noted in 6 animals treated with corticosteroids [prednisone (Δ_1 -Dehydrocortisone), cortisone, hydrocortisone] for 3-5 weeks nor in 3 rabbits in which hypercholesteremia had been induced.[†] In man, in several instances of multiple myeloma and liver disease a gamma glycoprotein band appeared in addition to the usual components and could be clearly separated from the O-fraction.

Discussion. In this study a quantitative pattern of glycoprotein distribution in the serum (plasma) of man, rabbit and dog has been established by paper electrophoresis. To our knowledge, no previous report of such a study is available. Other investigators(1, 4, 7-9) have reported qualitative patterns of serum glycoproteins in normal man and in various disease states(1,4). Some of these patterns differed from those obtained in this study in that a gamma-glycoprotein was described in normal serum. The carbohydrate-staining fraction with slowest mobility (or perhaps no mobility) in our experience corresponded to that material adsorbed at the point of application of the serum (O-fraction). It did not move with the gamma globulin which under our experimental conditions migrated "backwards," i.e., toward the cathode. In certain pathological states, however, a "true" gamma glycoprotein has been observed by others(1,4,7-9) and by ourselves. In all reports except that of Köiw and Gronwall, alpha-2 glycoprotein was the major component of the serum; in their graph, the largest glycoprotein peak was alpha-1.

The species examined showed distinctive patterns of glycoprotein distribution in a similar fashion to the species-specific protein and lipoprotein patterns described previously(2,3, 10,11). The glycoprotein distribution in man differed in a statistically significant manner from that obtained in dog and rabbit, with most marked divergence between man and rabbit. Previous examination of lipoproteins revealed a similar distribution pattern in man

[†] We are obliged to Dr. Chun-I Wang for providing these animals.

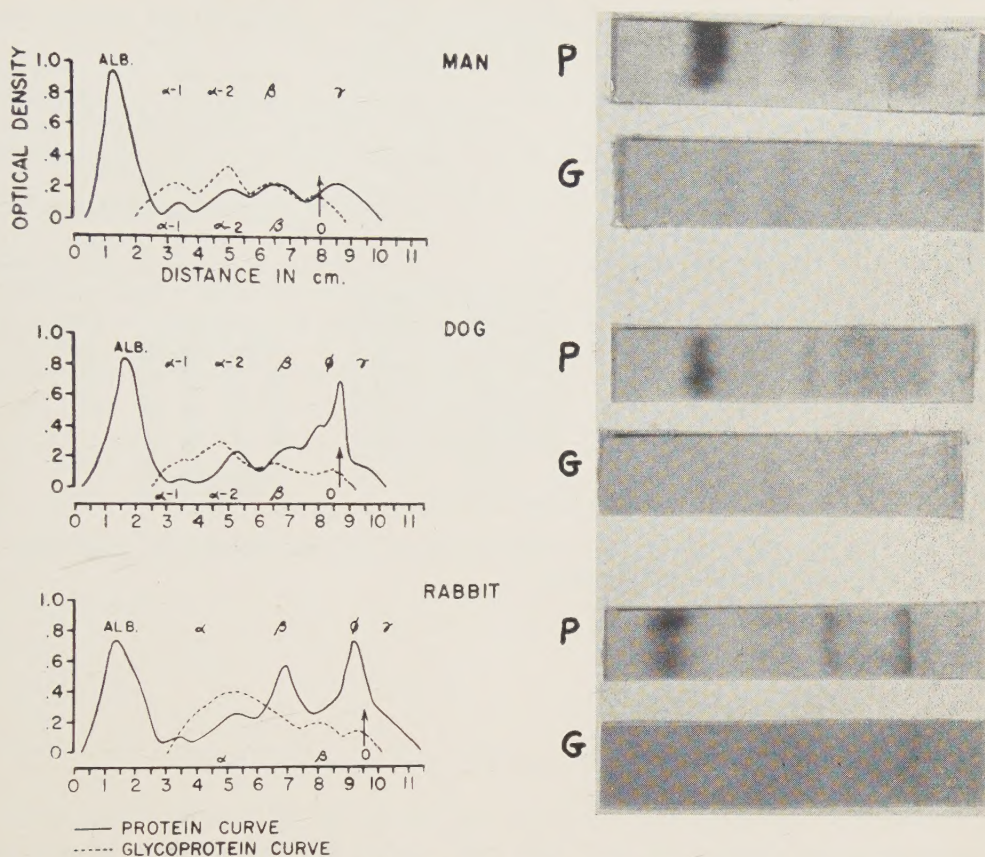


FIG. 1. Protein and glycoprotein electropherograms in man, dog and rabbit. On left are curves obtained by densitometric evaluation, on right are stained paper strips. P = Paper strip stained with amidoschwarz for protein. G = Paper strip stained with PAS for carbohydrate. Arrow in graphs and ruled line on paper strip represent the point of application of the serum (plasma). For details, see text.

and rabbit with regard to alpha and beta lipoprotein whereas in the dog the pattern was reversed. Thus, while species variations in these plasma components exist, they are not of parallel magnitude. Establishment of such normal patterns permits more detailed characterization of alterations in pathological states.

The technic of paper electrophoresis and subsequent periodic acid-Schiff stain did not yield values for distribution of serum glycoproteins comparable to those obtained by chemical analysis of plasma protein fractions (Cohn). The two procedures differed markedly and some discrepancy in results was not surprising; *e.g.*, alpha-1 glycoprotein appeared to be lower with the Cohn-fractionation technic. In addition, the analytical re-

sults of Cohn fractions differed with the author (12,13).

Summary. (1) By means of paper electrophoresis and subsequent carbohydrate staining by the periodic acid-Schiff method, quantitative evaluation of species glycoprotein patterns were established for man, dog and rabbit. (2) This procedure provides a simple, reproducible method for the quantitative study of serum glycoproteins which may be correlated with simultaneous determination of proteins and lipoproteins. (3) Carbohydrate was bound to alpha-1, alpha-2 and beta-globulins, with the major portion corresponding to the alpha globulins. Gamma glycoprotein was absent in normal serum in man. (4) No alterations were observed in rabbits after administration of adrenal cortical ster-

oids nor in experimentally-induced hypercholesteremia.

1. Köiw, E., and Gronwall, A., *Scand. J. Clin. and Lab. Invest.*, 1952, v4, 245.
2. Bossak, E. T., Wang, C. I., and Adlersberg, D., *PROC. SOC. EXP. BIOL. AND MED.*, 1954, v87, 637.
3. Adlersberg, D., Bossak, E. T., Sher, I. H., and Sobotka, H., *Clin. Chemistry*, 1955, v1, 18.
4. Rice, W. G., *J. Lab. and Clin. Med.*, 1954, v44, 544.
5. Jeanloz, K., *Science*, 1950, v111, 289.
6. Gomori, G., *Am. J. Clin. Path.*, 1952, v22, 277.
7. Osserman, E. F., and Lawlor, D. P., *Science*, 1954, v120, 715.
8. Sachs, B. A., Cady, P., and Ross, G., *Am. J. Med.*, 1954, v17, 663.
9. Kuhns, W. J., *J. Exp. Med.*, 1954, v100, 485.
10. Bossak, E. T., Wang, C. I., and Adlersberg, D., *PROC. SOC. EXP. BIOL. AND MED.*, 1955, v88, 634.
11. Bossak, E. T., Wang, C. I., and Adlersberg, D., *J. Clin. Endo. and Metab.*, in press.
12. Seibert, F. B., Pfaff, M. L., and Seibert, M. V., *Arch. Biochem.*, 1948, v18, 278.
13. Lever, W. F., and Hurley, N. A., *The Plasma Glycoproteins and Lipoproteins in Blood Cells, and Plasma Proteins*, edited by James L. Tullis.

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ERRATUM

In Vol. 88, 1955, article on rapid virulence test in diphtheria, page 369, line 8, 0.0045% should read 0.033%.